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**THE EFFECT OF THE ADMINISTRATION OF CALCIUM  
SALTS AND OF SODIUM PHOSPHATE UPON THE CAL-  
CIUM AND PHOSPHORUS METABOLISM OF THY-  
ROPARATHYROIDECTOMIZED DOGS, WITH A  
CONSIDERATION OF THE NATURE OF THE  
CALCIUM COMPOUNDS OF BLOOD AND  
THEIR RELATION TO THE PATHO-  
GENESIS OF TETANY.**

BY ISIDOR GREENWALD.

*(From the Harriman Research Laboratory, The Roosevelt Hospital, New York.)*

(Received for publication, October 6, 1925.)

INTRODUCTION.

In a previous communication (1), the author wrote:

"There are two, and only two, well authenticated metabolic changes after parathyroidectomy. One is the lowered calcium content of the serum or plasma and the other is the diminished excretion of phosphorus in the urine. These must be regarded as being intimately connected with the sequence of symptoms observed."

But the manner of such connection was not, at that time, satisfactorily evident.

It had been demonstrated by Salvesen (2) and by the author (3) that the retention of phosphorus is not, regularly, accompanied by an increase in the inorganic phosphate content of the plasma and Gross and Underhill (4) had shown that there is no regular increase in the total phosphorus of the blood.

More recently, it has been found (5) that the decreased calcium content of the serum is not due to an increased excretion of calcium, as had previously been believed.

Since the experiments to be described in the present communication were begun, Hanson (6), Berman (7), Collip (8), and Hjort, Robison, and Tendick (9) have made available a potent extract of beef parathyroids. The effect of the administration



of such an extract upon the excretion of nitrogen, phosphorus, calcium, and magnesium has been described and a theory of the pathogenesis of tetany following parathyroidectomy has been briefly presented (10).

It is the object of the present paper to present the results of experiments that were designed to determine (1) the effect of the administration of calcium salts upon the excretion of calcium and phosphorus in thyroparathyroidectomized dogs and (2) the effect of the injection of sodium phosphate upon thyroparathyroidectomized dogs in which tetany was being prevented by the administration of calcium salts.

In conclusion, an attempt will be made to correlate what seem to be the more significant observations on the tetany of parathyroidectomized animals and to show that they can all be explained by the hypothesis already alluded to; *viz.*, that the parathyroid hormone, or some substance to the preparation of which the parathyroid hormone is essential, maintains calcium in solution in the plasma in excess of the quantities possible in its absence. This hormone, or the substance derived from it or through its participation, is believed to form a compound with calcium that somewhat resembles calcium citrate in its general properties. The calcium is in non-ionic, but probably diffusible, form and is in equilibrium with ionic calcium in the solution. The beneficial effects of the various forms of treatment are believed to be due to their effect in increasing the amount of calcium in solution in the blood and other tissues.

#### EXPERIMENTAL.

The general conduct of the experiments and the methods of analysis were the same as in those already described (5, 10, 11).

In the first experiment of the present series, a fore period of 6 days was followed by a thyroparathyroidectomy. As soon as the fall in the ratio of phosphorus to nitrogen in the 24 hour urine had shown that the operation had been successfully performed, calcium chloride was injected intravenously. This was followed by vomiting but practically all of the vomit was caught and was later eaten. The rest of the vomit was analyzed with the urine and feces it had contaminated. No more calcium (other than that in the food) was administered for about 50 hours, when tetany

was observed. The injection of calcium chloride was followed by a complete remission of the tetany but the dog vomited. The vomit was eaten later, mixed with 30 gm. of calcium lactate. The same amount of calcium lactate was added to the food on the following day. It was then omitted and, on the 2nd day without calcium lactate, tetany again appeared. Calcium carbonate, *per os*, had no effect within 1.5 hours but an intravenous injection of calcium chloride produced immediate relief. For the following 7 days, the animal received additions of calcium chloride, carbonate, or lactate to the food, as indicated in Table I, and remained free from tetany, except on the 4th day, when an intravenous injection was required.

On the 8th day (the 15th since the operation), the serum contained 10.4 mg. calcium per 100 cc. Between 10 and 11 a.m. there were injected, intravenously, under cocaine anesthesia, 100 cc. of a solution of sodium phosphate, of pH 7.4, containing 1.11 gm. of phosphorus. There were no immediate untoward symptoms, certainly no sign of tetany. The usual diet, with the addition of 20 gm. of calcium lactate, was fed at noon. The dog vomited soon thereafter, the vomitus containing a large hair-ball. This may have been the cause of the vomiting. Tetany developed at about 1 p.m. and gradually became more severe. At 3.20 p.m., 20 gm. of calcium lactate were given with a stomach tube. The tetany gradually disappeared until, at 4 p.m., the dog was very lively and drank water eagerly.

For the next 5 days the animal received its usual food, with the addition of 20 gm. of calcium lactate. It remained free from tetany. The diet was then changed to 400 gm. of hashed beef heart and 30 gm. of calcium lactate, for each of 7 days, January 5 to 11. There was no tetany. The diet was then changed to the usual mixture of dried beef heart, cracker meal, and maize oil but with bone ash substituted for the infusorial earth. This was taken on the 1st day but refused on each of the 4 following, when it was given with a stomach tube. On the following day, January 17, 360 gm. of hashed beef heart and 15 gm. of infusorial earth were taken eagerly but, on the next day, they were refused. On the 19th, the original mixture of dried beef heart, cracker meal, maize oil, and infusorial earth was offered and refused. It was given with a stomach tube. The same was true for the

following 4 days, January 20, 21, 22, and 23. Slight twitching was noticed at 6 p.m., January 21 and again at 9 a.m., January 22, but not at 11 p.m. of the latter day. There was twitching all morning of the 23rd but the dog could walk about. At 2 p.m. the dog was found dead. *The limbs were not stretched out as in a dog dying from tetany.* An autopsy showed that death was probably due to an aspiration pneumonia. Blood was drawn from a jugular vein. It contained 8.17 mg. of inorganic and 26.2 mg. of "acid-soluble" phosphorus per 100 cc. The oxalated plasma contained 8.39 mg. of inorganic and 10.37 mg. of "acid-soluble" phosphorus per 100 cc. The serum contained 7.27 mg. of calcium per 100 cc.

The average amount of silica in the feces of the fore period was 11.7 gm. per day. In the much longer experimental period, the average was 11.34 gm. The difference is slightly greater than the error of the determinations. The high values for the amount of silica in the feces of the first 3 days of the fore period probably indicate that some feces had been carried over from the preliminary days. The values for the normal excretion of calcium and phosphorus in the feces are, therefore, probably about 3 per cent too high, but this does not affect the validity of the conclusions derived.

The next experiment was performed upon Dog 32 which had previously been used in experiments to determine the effect of the injection of calcium chloride and of sodium phosphate upon the excretion of nitrogen, phosphorus, calcium, and magnesium (10). Thyroparathyroidectomy was performed 8 days after the injection of sodium phosphate, before it was realized that the metabolism had not yet returned to normal. Consequently, the figures for the normal excretion that are presented in Table III have been taken from previous observations upon the same animal. The operation was followed by a marked decrease in the excretion of phosphorus. When the dog was seen about 13 hours after the operation, it presented a very excited appearance. It seemed as if it had recently had a short attack of tetany, one not sufficiently severe to leave the dog exhausted. The next morning, a short attack of tetany was observed and 50 gm. of calcium lactate were added to the food. The same addition was made on each of the following 5 days. The calcium lactate caused

a diarrhea which made complete separation of urine and feces impossible. The low figures for the nitrogen in the urine of these days are probably due chiefly to loss of nitrogen in the feces due to defective absorption. The general condition of the dog seemed very good. There was no sign of tetany. On the 6th day of the administration of calcium lactate, about 4 hours after feeding, a femoral vein was exposed under cocaine anesthesia. Blood serum obtained at this time contained 11.1 mg. of calcium per 100 cc. The oxalated plasma contained 2.76 mg. of inorganic, and 3.55 mg. of "acid-soluble" phosphorus per 100 cc. The former value may be too low because of the presence of an excess of oxalate. The withdrawal of blood was followed by the intravenous injection of 70 cc. of sodium phosphate solution, of pH 7.4, containing 0.778 gm. of phosphorus. Although the dog was observed almost continuously to 6 p.m. and again at 8.15 and 10 p.m., absolutely no untoward symptoms were seen. The amount of calcium lactate was reduced on the next day and none at all was given on the 2nd day. On the 3rd day, tetany appeared. This persisted and grew worse after feeding the usual diet with the addition of 10 gm. of calcium lactate, but was relieved by the intravenous injection of calcium chloride. Blood was drawn just before the injection. The serum contained 5.55 mg. of calcium per 100 cc. and the plasma 3.41 mg. of inorganic phosphorus per 100 cc.

On the following day, March 29, 10 gm. of calcium lactate were added to the usual ration. About one-half of the food was eaten; the rest was given with a stomach tube. On the 30th, the usual ration plus 10 gm. of calcium lactate was offered and refused. A stomach tube was used but the dog vomited about one-third of the food immediately upon the withdrawal of the tube. About 4 hours later, a sample of blood was drawn. The serum contained 6.11 mg. of calcium per 100 cc. The plasma contained 4.17 mg. of inorganic and 4.81 mg. of "acid-soluble" phosphorus per 100 cc. 70 cc. of the same phosphate solution as had been used 6 days previously were injected. The dog drank considerable water immediately after the injection but vomited about 3 hours later. There were no other symptoms up to at least 4 hours after the injection. The dog was not observed again for 4 hours and was then found in tetany. After drawing

samples of blood, the dog was killed. The serum contained 6.11 mg. of calcium per 100 cc. and the plasma 6.28 mg. of inorganic and 6.95 mg. of "acid-soluble" phosphorus per 100 cc.

The corrected value for the silica content of the feces of the control periods was 18.78 gm. per day. The average for the period following thyroparathyroidectomy was 18.93 gm., if the last day upon which only a small amount of feces were obtained, is omitted. The difference is within the error of the determination.

#### DISCUSSION.

*Retention of Phosphorus and Calcium.*—The results of the daily analyses of the urine and feces of these animals are summarized in Tables I and III, respectively. Even cursory examination of these shows that there was, after thyroparathyroidectomy, a marked retention of both calcium and phosphorus. For the purpose of making this more evident and in order to show more clearly the rate of retention, Tables II and IV have been prepared. In these, the first column gives the date and the next two columns the excretion of phosphorus and calcium from the day of the operation to and including that date. For instance, in Table II, the total excretion of phosphorus on December 16, the day of the operation, was 0.295 gm. and the total excretion of calcium was 0.051 gm. The next *horizontal* line has the date, 17, followed by the total amount of phosphorus and calcium, 0.402 and 0.094 gm., respectively, excreted on the 16th and 17th, combined. And so on down the table. The next two vertical columns contain the sum of the additional amounts of calcium and phosphorus administered and the product of the daily normal excretion by the number of days elapsed since the operation. The last two vertical columns contain, respectively, the difference between Columns 4 and 2 and between Columns 5 and 3. They indicate the amounts of phosphorus and of calcium that had been retained to the given date. The values are somewhat too high, for the calcium excretion, particularly, was higher during the fore period than was the intake. But even if the basal diet had contained no calcium whatever, the retention of calcium would still be very large.<sup>1</sup>

<sup>1</sup> The materials used in these experiments were not analyzed but determinations made on other specimens indicate that the food of Dog 29 contained 0.308 gm. of phosphorus and 0.050 gm. of calcium per day while that of Dog 32 contained 0.485 gm. of phosphorus and 0.080 gm. of calcium.

The determinations of the calcium in the urine and feces of Dog 29 on December 29 were lost. From a consideration of the amounts of urine and feces passed on this day and the concentration of calcium in the urine and feces of the preceding and following days, it appears that the total amount of calcium in the excreta of December 29 was about 1 gm. This loss has not been included in Tables I and II and, consequently, all the subsequent figures for the "retention" of calcium are too high by this amount.

Salvesen (2) had reported that, in parathyroidectomized dogs, injected calcium was promptly and completely excreted. At the time that these experiments were begun, it was believed that they would confirm Salvesen's report and that the beneficial effect of the administration of calcium salts would be found to be accompanied by an increased excretion of phosphorus. Instead, a marked retention of both phosphorus and calcium was observed. Obviously, this retention of increasing amounts of calcium cannot continue indefinitely. Sooner or later, a balance between intake and output must be reached. This seems to have occurred after about the 15th day in the experiment upon Dog 29 and the 10th day in that upon Dog 32. Salvesen's experiments were performed after calcium salts had been administered for 11, 13, and 6 days, respectively. It was in the two former experiments that the prompt, complete excretion of the injected calcium was observed. In the third experiment, only 0.172 gm. out of the 0.578 gm. injected were excreted.

It is interesting to observe that, whenever the administration of calcium was interrupted or the amount was reduced with the resultant appearance of tetany, the amount of "retained" calcium was, except on December 18, the 3rd day of the first experiment, about four times as great as the amount of phosphorus. Even assuming that the food contained no calcium and deducting the amount excreted from the amount of additional calcium administered as calcium chloride, carbonate, or lactate, the amount of retained calcium was about three times as great as the amount of retained phosphorus. The ratio of calcium to phosphorus in  $\text{Ca}_3(\text{PO}_4)_2$  is 1.94. It might have been expected that the excess of calcium over that required to form  $\text{Ca}_3(\text{PO}_4)_2$  with the retained phosphorus would have been available to maintain the calcium content of the plasma and serum but, apparently, this was not the case. The excess of calcium must have been

TABLE I.  
*Excretion of Phosphorus and Calcium As Influenced by Thyroparathyroidectomy and Administration of Calcium Salts and Sodium Phosphate.*

Date. 1924	Urine.				Feces.			Remarks.
	Nitrogen.	Phos- phorus.	Calcium.	Silica.	Phos- phorus.	Calcium.		
						gm.	gm.	
Dec.								
10	4.37	0.345	0.0002	12.8	0.111	0.187		
11	3.97	0.324	0.0002	13.8	0.099	0.158		
12	3.92	0.323	0.0002	15.3	0.099	0.159		
13	4.01	0.308	0.0001	8.8	0.071	0.112		
14	3.52	0.281	0.0002	3.6	0.032	0.057		
15	5.32	0.458	0.0003	16.1	0.115	0.179		
Average.	4.185	0.340	0.0002	11.7	0.088	0.142		
16	5.00	0.251	0.003	5.3	0.044	0.051	Thyroparathyroidectomy 10.30 a.m.	
17	4.43*	0.071	0.0002	6.3	0.036	0.043	Injected 18 cc. $\text{CaCl}_2$ = 0.441 gm. Ca. Vomited. Most of vomit eaten later; rest in urine.	
18	4.70	0.169	0.0014	10.6	0.086	0.093	1.45 p.m. tetany. Injected 17 cc. $\text{CaCl}_2$ = 0.416 gm.	
19	2.62†	0.010	0.333	18.3	0.146	0.307	Ca. Vomited. Vomit eaten later, with 30 gm. Ca lactate.	
20	5.32†	0.225	1.31				30 gm. Ca lactate added to food.	
21	3.44	0.050	0.0001	14.7	0.165	0.778	1 p.m. tetany. 5 gm. $\text{CaCO}_3$ per os; no effect. In-	
22	3.42	0.070	0.0223	21.7	0.134	0.441	jected 12 cc. $\text{CaCl}_2$ (= 0.294 gm. Ca). Relief.	

23	2.93	0.012	0.0187	7.9	0.075	0.988	5 gm. CaCO <sub>3</sub> with food.
24	2.73	0.029	0.0156	24.8	0.490	3.420	5 gm. CaCO <sub>3</sub> and 20 cc. CaCl <sub>2</sub> (= 0.490 gm. Ca) with food.
25	3.54	0.074	0.0007	8.0	0.154	0.641	20 cc. CaCl <sub>2</sub> (= 0.490 gm. Ca) with food.
26	2.68	0.093	0.0004	7.3	0.091	0.220	9.30 a.m. slight twitching, fed usual mixture 20 cc. CaCl <sub>2</sub> (= 0.490 gm. Ca). Twitching worse at 10.20 but absent at 12.30. 4.30 p.m. marked tetany. Injected 9 cc. CaCl <sub>2</sub> (= 0.211 gm. Ca). Immediate relief.
27	2.90	0.008	0.0010	9.8	0.131	0.348	10 gm. Ca lactate with food.
28	3.49	0.045	0.272	12.6	0.246	1.425	10 " " " "
29	1.86	0.005	Lost.	3.5	0.081	Lost.	20 " " " "
30	3.63*	1.186	0.0010	12.0	0.228	2.17	†
31	2.35	0.116	0.0005	9.1	0.191	3.71	20 gm. Ca lactate with food.
Jm.							
1	3.51	0.010	0.0005	17.1	0.318	3.73	20 " " " "
2	3.09	Trace.	0.0004	11.8	0.263	2.80	20 " " " "
3	5.38	"	0.0003	6.8	0.105	0.972	20 " " " "
4	6.06	"	0.0002	19.3	0.395	4.02	20 " " " "
Average.	3.654			11.34			

\* Contaminated with vomit.

† Contaminated with feces.

‡ 10 a.m., drew 50 cc. blood from femoral vein. Whole blood contained 4.09 mg. inorganic and 19.1 mg. total "acid-soluble" P per 100 cc. Plasma contained 4.22 and 4.32 mg., respectively. Serum contained 10.4 mg. Ca per 100 cc. 10-11 a.m., injected 100 cc. Na phosphate, pH 7.4, containing 1.112 gm. P into femoral vein. No symptoms. Fed usual mixture, with 20 gm. Ca lactate at noon. Vomited soon thereafter, the vomitus containing a large hair-ball. Developed tetany about 1 p.m. T: is gradually became more severe. At 3.20 p.m. 20 gm. Ca lactate by stomach tube. 4 p.m., no tetany. Dog lively.



*Dogs. Comparison of normal excretion and the additional amounts administered.*  
 Dog 29. See Table I and text p. 3.

Date.	Excreted.		Normal excretion + additional phosphorus and calcium administered.		Retained.		Remarks.
	Phosphorus.	Calcium.	Phosphorus.	Calcium.	Phosphorus.	Calcium.	
1924	gm.	gm.	gm.	gm.	gm.	gm.	
Dec.							
16	0.295	0.051	0.428	0.142	0.133	0.091	
17	0.402	0.094	0.856	0.725	0.454	0.631	
18	0.657	0.189	1.284	0.867	0.627	0.678	
19	0.813	0.829	1.712	5.325	0.899	4.496	Tetany, injected $\text{CaCl}_2$ .
20	1.038	2.139	2.140	9.225	1.102	7.086	
21	1.253	2.917	2.568	9.367	1.315	6.450	
22	1.457	3.380	2.996	11.80	1.539	8.439	Tetany, injected $\text{CaCl}_2$ .
23	1.544	4.387	3.424	13.95	1.880	9.558	
24	2.063	7.823	3.852	16.58	1.789	8.754	
25	2.291	8.464	4.280	17.21	1.989	8.745	Tetany, injected $\text{CaCl}_2$ .
26	2.475	8.684	4.708	18.05	2.233	9.368	
27	2.614	9.033	5.136	19.49	2.52	10.46	
28	2.905	10.73	5.564	20.93	2.66	10.20	
29	2.991	10.73*	5.992	24.17	3.00	13.44†	
30	3.405	12.90*	7.532	29.51	4.13	16.61†	Injected $\text{PO}_4$ solution. Tetany. Injected $\text{CaCl}_2$ .
31	3.712	16.71*	7.960	32.25	4.25	15.54†	
Jan.							
1	4.040	20.44*	8.388	33.00	4.35	12.56†	
2	4.303	23.24*	8.816	35.74	4.51	12.50†	
3	4.408	24.21*	9.244	38.48	4.83	14.27†	
4	4.803	28.23*	9.672	41.22	4.87	12.99†	

deposited as some insoluble and, for this purpose, unavailable compound. In this connection, the report by Jolibois and Maze-Sencier (12) of the large amounts of  $\text{Ca}(\text{OH})_2$  that may be adsorbed by  $\text{Ca}_3(\text{PO}_4)_2$  precipitated in the presence of excess of  $\text{Ca}(\text{OH})_2$  is of considerable interest. Something of the same sort may have occurred in the body.

That this calcium was, however, available for other purposes is made probable from the following considerations. During the 6 days following the injection of sodium phosphate, the amount of calcium retained by Dog 29 was not increased at all. During these same 6 days, 1.87 gm. of phosphorus were added to the body. It is not unreasonable to assume that this phosphorus was combined with the calcium previously stored in excess of the quantities required to form  $\text{Ca}_3(\text{PO}_4)_2$ .

In the experiment upon Dog 32, large amounts of calcium lactate were fed. This seemed to stimulate intestinal peristalsis, as evidenced by the diarrhea. The low values for urinary nitrogen are probably due to defective absorption of food. Some phosphate probably similarly escaped absorption and more was probably precipitated and made relatively non-available by the large amounts of calcium. Whatever the explanation may be, the retention of phosphorus was 0.432 gm. on the 1st day, before calcium lactate was fed, and only about 0.050 gm. on each of the following 5 days. The amount of calcium retained was far in excess of the quantity required to form  $\text{Ca}_3(\text{PO}_4)_2$ .

No attempt has, as yet, been made to determine the location of these deposits of calcium and phosphorus. Leopold and von Reuss (13) found that the combined soft tissues of parathyroidectomized rats contained a greater concentration of calcium than did those of control rats, whereas the skeletons of both sets of animals had about the same concentration of calcium. The retained calcium and phosphorus are, apparently, not available for either normal tooth or bone formation for Erdheim (14), Iselin (15) and Toyofuku (16) found that the teeth of parathyroidectomized rats were not properly calcified. Korenchevsky (17) also observed defective dentition, though not so marked as in Erdheim's experiments, in parathyroidectomized rats. A similar defective calcification of the bones was reported by Erdheim (14) and Canal (18) and Morel (19) found that the bones of parathyroidectomized rats did not knit normally after fracture.

TABLE II.  
*Retention Phosphorus and Calcium after Thyroparathyroidectomy and after Administration of Calcium Salts. Total Amounts Excreted up to the Given Date with Those Calculated from the Normal Excretion and the Additional Amounts Administered.*

Dog 29. See Table I and text p. 3.

Date.	Excreted.		Normal excretion + additional phosphorus and calcium administered.		Retained.		Remarks.
	Phosphorus.	Calcium.	Phosphorus.	Calcium.	Phosphorus.	Calcium.	
1924	gm.	gm.	gm.	gm.	gm.	gm.	
Dec.							
16	0.295	0.051	0.428	0.142	0.133	0.091	
17	0.402	0.094	0.856	0.725	0.454	0.631	
18	0.657	0.189	1.284	0.867	0.627	0.678	
19	0.813	0.829	1.712	5.325	0.899	4.496	Tetany, injected CaCl <sub>2</sub> .
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30	3.405	12.90*	7.532	29.51	4.13	16.61†	Injected PO <sub>4</sub> solution. Tetany. Injected CaCl <sub>2</sub> .
31	3.712	16.71*	7.960	32.25	4.25	15.54†	
Jan.							
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3	4.408	24.21*	9.244	38.48	4.83	14.27†	
4	4.803	28.23*	9.672	41.22	4.87	12.99†	

\* No calcium determinations. Dec. 29. All figures too low by about 1 gm.

† No calcium determinations. Dec. 29. All figures too high by about 1 gm.

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TABLE IV.  
*Retention of Phosphorus and Calcium after Thyroparathyroidectomy and Administration of Phosphate and Calcium Salts.  
 Comparison of Amounts Excreted to Any Given Date with Those Calculated from the Normal Excretion and the Addi-  
 tional Amounts Administered.*

Dog 32. See Table III and text p. 11.

Date. 1925	Excreted.		Normal excretion + extra amount administered.		Retained.		Remarks.
	Phosphorus.	Calcium.	Phosphorus.	Calcium.	Phosphorus.	Calcium.	
<i>Mer.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
19	0.098	0.171	0.530	0.293	0.432	0.122	Thyroparathyroidectomy. Short attack of tetany.
20	0.587	6.44	1.060	7.09	0.473	0.65	
21	1.067	12.27	1.590	13.88	0.523	1.61	
22	1.515	18.23	2.120	20.67	0.605	2.44	
23	2.020	24.72	2.650	27.47	0.630	2.75	Injected 0.778 gm. P as sodium phosphate.  Tetany relieved by injection of 0.245 gm. Ca as CaCl <sub>2</sub> .
24	2.513	31.84	3.180	34.26	0.667	2.42	
25	3.109	31.84	4.488	41.06	1.379	9.22	
26	3.900	39.58	5.018	44.59	1.118	5.01	
27	3.929	39.60	5.548	44.89	1.619	5.29	
28	4.261	40.31	6.078	46.73	1.817	6.42	
29	4.954	42.78	6.608	48.33	1.654	5.55	

It is possible that the cataract so frequently observed in parathyroidectomized dogs (for literature see Biedl (20)) and in cases of tetany in man (for literature see Barker (21)) may be due to deposition of calcium phosphate in the lens.

*Relation of Phosphate Retention to Tetany.*—In 1911, it was shown (22) that the decreased excretion of phosphorus in the urine first observed by Ver Eecke (23) after thyroparathyroidectomy was due to the removal of the parathyroids and bore no relation to the thyroid deficiency. It was also found that this retention of phosphorus was not accompanied by an increased excretion of phosphorus into the feces. Because of the striking character of this retention, attempts were made to correlate it with the appearance of the symptoms.

Injection of solutions of sodium phosphate did not produce tetany (24). Binger (25) later claimed to have produced tetany in this manner but it was subsequently (26) shown that the same sort of convulsions as were produced by the injection of sodium phosphate were also to be observed after the injection of other sodium salts. Larger amounts were required, it is true, but that was, probably, chiefly because these other salts were more rapidly excreted by the kidneys. *At the time convulsions appeared, the concentration of sodium in the plasma was approximately the same, no matter whether the phosphate, chloride, sulfate, or carbonate was employed* (27).

Tisdall (28) also claimed to have observed tetany in dogs after the injection of sodium phosphate but not after the administration of an equivalent quantity of phosphoric acid, although the changes in the phosphorus and calcium content of the serum were the same in both cases. He also claimed that the injection of sodium phosphate was not followed by an increase in the concentration of sodium in the serum, without offering any explanation for the disappearance of the sodium. In a personal communication to the author, he has admitted that his sodium determinations were probably at fault.

Underhill, Gross, and Cohen (29) found that both the acid and alkaline phosphates produced tetany, the amount required being dependent upon the amount of base introduced with the phosphate ion.

Salvesen, Hastings, and McIntosh (30) produced tetany by



the repeated oral administration of large amounts of sodium phosphate. In their experiments, the increase in the sodium content of the plasma was negligible, the change in the chlorine content was similarly slight, and there can be little doubt but that the tetany was really due to the removal of calcium: not merely to the observed diminution of the amount of calcium in the serum, but also to the accompanying, or even preceding, removal of active calcium from other tissues. The fate of the calcium that disappeared from the plasma was not determined.

But these observations did not help materially to elucidate the relation between the retention of phosphate after parathyroidectomy and the development of tetany. And, indeed, as long as MacCallum and Voegtlin's report (31) of an increased excretion of calcium was accepted, it was impossible to do so for, as has already been stated, it had been shown that there was, after parathyroidectomy, no increase in the excretion of phosphorus in the feces.

With the discovery that the removal of the parathyroids (and thyroids) led to a diminished excretion of calcium (5) and that the administration of a potent parathyroid extract was followed by an increased excretion of both calcium and phosphorus (10), the way was opened to what seems to be a satisfactory explanation for the pathogenesis of tetany.

#### *The Nature of the Calcium in the Plasma and Its Relation to the Pathogenesis of Tetany.*

It has long been known that the plasma and serum contain calcium in excess of the amount that an aqueous solution of the same reaction and phosphate content could keep in solution. Holt, La Mer, and Chown (32) have recently shown that when serum is shaken for a long time with solid  $\text{Ca}_3(\text{PO}_4)_2$ , the concentration of both calcium and phosphate in solution diminishes. Thus, serum acts as a supersaturated solution of calcium phosphate, and Holt, La Mer, and Chown so regard it. They postulate the presence, in serum, of a substance which delays the precipitation of calcium absorbed from the intestine.

But such a hypothesis, alone, offers no explanation for the maintenance of the normal, or nearly normal, concentration of

calcium in cases of calcium deficiency, particularly in pregnancy and lactation, when large amounts of calcium may be removed from the bones. It seems to be necessary to assume the existence of some organic substance capable of combining with calcium, not only so as to keep it from being precipitated by the phosphate of the plasma but also so as to dissolve it from the calcium phosphate of the bones. It would seem that the parathyroid hormone is this substance or, at least, is essential to its preparation.

Before proceeding to a more complete discussion of this hypothesis, it seems advisable to discuss the somewhat similar theory of Cameron and Moorhouse (33) and the evidence upon which the latter is based. These authors found that the concentration of calcium in the cerebrospinal fluid of normal dogs was about 53 per cent of that in the serum. They regard the concentration in the cerebrospinal fluid as identical with that of the diffusible calcium of the plasma and conclude that the other 47 per cent is combined with some organic constituent. This view they believe to be supported by the fact that, when diluted blood was treated with oxalate and rapidly centrifuged, about 2 mg. of calcium per 100 cc. of blood were found in the supernatant liquid. This precipitated only very slowly.

The same small amount of slowly precipitable calcium was found in the plasma of dogs in tetany after thyroparathyroidectomy. In such dogs, the calcium content of the whole blood was as high as that of the serum. The concentration of calcium in the citrated plasma, after allowing for the changes in volume due to the citrate, was greater than that of the serum, although, in normal dogs, the values were generally the same. (In one experiment out of three on normal dogs, the difference was 3 mg. Two samples of blood from the same dog in tetany gave differences of 2.7 and 2.9 mg., respectively, and the average of the determinations on seven samples of blood from dogs in tetany was 3.1 mg.) The concentration of calcium in the cerebrospinal fluid of dogs in tetany was from 70 to 100 per cent of that in the serum. Although non-diffusible calcium was, apparently, entirely lacking from the sera of some of these animals, the oxalated *plasmas* contained as much slowly precipitable calcium as did those of normal dogs.

Cameron and Moorhouse interpret these results as indicating that the amount of the organic calcium compound in the blood is diminished after thyroparathyroidectomy and that such as is present becomes bound to the clot, instead of being liberated in the serum as is the case with normal blood.

Whatever the factors that maintain calcium in solution in serum in excess of the quantities possible in a simple salt solution of the same hydrogen ion, bicarbonate, and phosphate concentration may be, they may reasonably be expected to similarly interfere with the complete precipitation of calcium by means of oxalate. Cameron and Moorhouse do not state how much oxalate they added to the whole blood before centrifuging to recover the slowly precipitating calcium in the plasma. Presumably, they used only 0.1 per cent, which is the amount ordinarily employed. This is five or six times the equivalent of the amount of calcium present in blood. In the method employed by Cameron and Moorhouse for the direct precipitation of calcium from serum (Tisdall (34)), the amount of oxalate employed is forty times as great as the equivalent of the amount of calcium to be precipitated. The difference in the amount of excess oxalate used may well explain the difference in the rate of precipitation and calcium from whole blood and serum. Moreover, it is possible that the formed elements of the blood also contain the substance, or substances, that keeps calcium in solution in the serum. The excess of oxalate required to rapidly and completely precipitate the calcium from blood would then be even greater than that required for serum.

From their determinations of the calcium content of serum and whole blood, Kramer and Tisdall (35) concluded that normal human erythrocytes do not contain calcium. Employing lithium citrate as an anticoagulant, Stanford and Wheatley (36) analyzed whole blood, plasma, and the centrifuged corpuscles. They found from 1.4 to 3.2 mg. of calcium per 100 cc. of cells in the five samples of human blood examined. Whatever may be the case in normal, human blood, there is no justification for assuming that the cells of the blood of dogs in tetany do not contain calcium. If the concentration of calcium in the whole blood is as high as it is in the serum, as Cameron and Moorhouse claim, the obvious explanation is that the cells contain appreciable amounts of

calcium. When such blood is citrated, all of the calcium in the plasma is made non-ionic and the calcium in the cells may be expected to diffuse out. This would explain the results obtained.

Unpublished experiments, in which heparin was used as the anticoagulant, showed no difference between the calcium content of the plasma and that of the serum of thyroparathyroidectomized dogs.

There is, therefore, in the work of Cameron and Moorhouse no satisfactory evidence of the presence of an organic compound specifically related to parathyroid function. The work of others seems to make such an hypothesis necessary. But, because they accepted the views of MacCallum and Voegtlin (31) and of Salvesen (2), Cameron and Moorhouse were led to conclude that the organic constituent prevents the excretion of calcium. In its absence, as after parathyroidectomy, inorganic calcium, in their opinion, escapes through the intestinal epithelium. But exactly the opposite is really the case.

In view of the facts that have been developed, it seems to be necessary to assume:

1. That the amount of ionic calcium in the blood is always small, except after the administration of large amounts of calcium salts. This ionic calcium is in equilibrium, at the bones, etc., with solid  $\text{Ca}_3(\text{PO}_4)_2$  and, in the circulating blood, with:

2. At least one organic compound of calcium. The one of significance in relation to the function of the parathyroids resembles calcium citrate in certain of its properties. It liberates very little of its calcium as calcion and is not readily precipitated by oxalate or by phosphate. Unpublished experiments in this laboratory have shown that, in the presence of two equivalents of sodium citrate, a solution of calcium chloride containing 10 mg. of calcium per 100 cc. is not completely precipitated by even 5 equivalents of sodium oxalate in 24 hours. A solution containing 10 mg. of calcium, 2 equivalents of sodium citrate, and 2 of sodium phosphate remains perfectly clear, indefinitely, at pH 7.4. However, the hypothetical substance is not sodium citrate (37).

3. An equilibrium between these two forms of calcium. The precise distribution of calcium between the two forms is determined by the concentration of calcium, of the organic constituent,

of phosphate, carbonate, or other ions tending to precipitate calcium, hydrogen ion, etc. Because of this equilibrium between the two forms of calcium, it is impossible, at present, to regard the rate of excretion of calcium as, normally, a function of either one alone.

Assuming that the parathyroid hormone is this organic substance, which we will call  $X$ , or, more probably, is necessary to its preparation, let us consider the effect of changes in the amount of parathyroid secretion and of the administration of various substances to normal and to parathyroidectomized dogs.

Administration of parathyroid extract to a normal dog displaces the equilibrium  $\text{CaX} \rightleftharpoons \text{Ca}'' + X$  to the left. The decreased concentration of  $\text{Ca}''$  in the blood displaces the equilibrium  $\text{Ca}'' + {}''\text{PO}_4 \rightleftharpoons \text{Ca}_3(\text{PO}_4)_2$  at the bones, also to the left. The concentrations of calcium and of phosphate in the plasma are increased (38). There is, in consequence, an increased excretion of calcium and of phosphate (10). The paths of excretion for both phosphates and calcium depend upon the reaction of the blood.

It is interesting to observe, in this connection, that hypertrophy or hyperplasia of the parathyroid glands has been found in osteomalacia (39) and in rats on a diet deficient in calcium (40).

Removal of the parathyroids leads to prompt diminution in the amount of  $X$ . Consequently, the equilibrium  $\text{CaX} \rightleftharpoons \text{Ca}'' + X$  is displaced to the right. So is the equilibrium  $\text{Ca}'' \rightleftharpoons \text{Ca}'' + \text{Ca}_3(\text{PO}_4)_2$ . The concentration of calcium in the plasma falls and the concentration of phosphate would be similarly decreased if it were not maintained by that derived from the food and from the endogenous metabolism. But the excretion of both calcium and phosphorus decreases (5); that of the latter to the greater extent because it furnishes all the phosphate that is deposited whereas the calcium deposition takes place chiefly at the expense of the calcium of the blood. As the concentration of  $\text{CaX}$  diminishes, it is probable that other equilibria are upset and that calcium escapes from other tissues. It is probably the loss of  $\text{Ca}''$  from certain of these, particularly the neuromuscular junction (41), rather than the change in the blood itself, that is the cause of tetany.

From the data summarized by Holt, La Mer, and Chown and

from their own experiments (32), it is evident that the amount of ionized calcium, at equilibrium, in the plasma cannot be more than 2.5 mg. per 100 cc. Neuhausen and Marshall (42), employing a calcium electrode, found from 2.1 to 2.3 mg. of calcium per 100 cc. of serum. Brinkman and Van Dam (43), by determining the concentration of oxalate required to produce a precipitate, found from 2 to 3 mg. calcium per 100 cc. of the ultrafiltrate from serum. Since the concentration of diffusible calcium in serum is much greater than this (Rona and Takahashi (44), von Meysenbug, Pappenheimer, Zucker, and Murray (45), Moritz (46)), the existence of a diffusible, non-ionized, calcium compound is very probable.

The concentration of calcium in the serum of parathyroidectomized animals never falls to as little as 2 or 3 mg. per 100 cc. It may be that there are organic compounds of calcium that have no relation to parathyroid function; it may be that the parathyroid hormone, or substance derived from it or through its agency, never entirely disappears. Possibly both are true.

The results of Salvesen and Linder (47), who found that in cases of nephritis without phosphate retention the decrease in the concentration of calcium in the serum paralleled the decrease in the concentration of protein, would seem to indicate that at least 3 and, probably, as much as 5 mg. of calcium are, normally, combined with protein. Calcium thus combined seems to have no intimate relation to the functioning of the parathyroid glands.

Accessory parathyroid glands have been found so often as to make it very probable that they exist even more frequently. The fact that animals that have been kept alive by means of calcium administration, etc., may later remain free from tetany even though all dietary control has ceased indicates very strongly that there are accessory parathyroids in such animals or that some other tissue may take over the function of the parathyroid glands. The existence of such an animal as Salvesen's Dog 2, which could be brought into tetany by a calcium-poor (and phosphate-rich) diet as long as 20 months after parathyroidectomy is very good evidence that the resistance of such animals as develop it is due to the presence of accessory parathyroid glands. There is no apparent reason why, in this animal, the

other tissues that might be supposed to take over parathyroid function did not do so. It is more reasonable to suppose that this dog had no accessory parathyroid tissue or, at least, that this did not hypertrophy sufficiently to replace that which had been removed.<sup>2</sup>

The periods of remission from tetany that so frequently occur, even without treatment, also seem to point to the presence of accessory parathyroids. Recovery from tetany may be due to stimulation of such accessory glands by metabolites formed during tetany or it may be due to the action of such acid metabolites in increasing the excretion of acid phosphates, thus making more calcium available. Certainly, attacks of tetany are frequently followed, or accompanied, by increased excretion of phosphate (22). The continuance of the remission depends upon the functioning of the accessory parathyroid tissue.

When calcium salts are administered by mouth, part of the calcium is absorbed as calcion. When injected as the lactate or chloride, all of the calcium is present as calcion. Either, directly, by virtue of the increased concentration of calcion or, indirectly, because this displaces the equilibrium  $\text{CaX} \rightleftharpoons \text{Ca}'' + \text{X}$  to the left, the administration of calcium restores the calcium content of the essential tissues and tetany disappears. As is evident from the results reported in this paper, much of the calcium administered may be deposited as calcium phosphate and in other forms. But, eventually, this deposition stops. Perhaps all the available places become saturated. Thereafter, only as much calcium as is lost need be supplied to keep the animal free from tetany. Even if the amount of calcium in the food is insufficient to maintain a calcium balance, some of the deposited calcium may become available. This may explain why Dog 29 did not develop tetany after his saturation with calcium until after 5 days on which the calcium addition was in the form of bone ash and until after 5 more days on a low calcium diet. Our own experience confirms the reports of Luckhardt and Goldberg (48) and of Compere and Luckhardt (49) that administra-

<sup>2</sup> Blum (Blum, F., Studien über die Eipthelkörperchen, Jena, 1925) has reported several similar instances in cats. A change of diet, a year or more after the removal of the parathyroids, was followed by the appearance of tetany.

tion of bone ash, soon after parathyroidectomy, will not prevent tetany. But, after the calcium deposits have become filled, the small amounts of calcium available from bone ash may be sufficient to maintain the animal free from tetany.

The effect of the administration of phosphate into a parathyroidectomized dog that has been kept free from tetany by means of the administration of calcium salts, will depend upon the dose and upon the store of calcium not combined with phosphate. If the latter is large, as it seems to have been in Dog 32 on March 25, the animal shows no more symptoms after the administration of 50 mg. of phosphorus per kilo of body weight than does a normal dog. But if the store of available calcium is low, as in Dog 32 on March 30, or if the dose is very large, as in Dog 29, the disturbance in the equilibria in the blood brought about by the injection of the phosphate may be sufficient to withdraw enough calcium from the essential tissues to cause tetany, before the injected phosphate can be excreted. The injurious action of acid calcium phosphate (49) is, evidently, due to the excess of phosphoric acid.

In a normal dog, the injection of calcium chloride leads to an increased excretion of calcium in both urine and feces. This is quite in agreement with the theory here advanced. The increased concentration of calcium leads to an increased concentration of  $\text{CaX}$  and one, or both, stimulate the excretion of calcium. But why should the simultaneous injection of sodium phosphate with the calcium chloride lead to a still greater excretion of calcium (10)? The concentration of  $\text{Ca}^{++}$  and, consequently, that of  $\text{CaX}$ , in the blood might be expected to be lower in the presence of the sodium phosphate. There are, however, many other factors that may be involved, such as changes in reaction due to differences in the speed with which the various ions  $\text{Ca}^{++}$ ,  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{H}_2\text{PO}_4^-$ , and  $\text{HPO}_4^{--}$  are excreted; the possible effects of any one or more of these ions upon the formation and liberation of  $\text{X}$ , etc.

In the experiments of Salvesen, Hastings, and McIntosh (30), to which reference has already been made, the lowered concentration of calcium in the serum may have been due to precipitation of calcium and deposition in certain tissues or to an increased excretion of calcium. In either case, the calcium con-



tent of the plasma and of other tissues would be depleted. In their first experiments, the drain on the body's store of calcium was not long continued and no tetany resulted. With more prolonged administration of phosphates, the depletion of calcium became sufficiently great to cause tetany.

According to Salvesen (2), the injurious action of a meat diet in parathyroidectomized dogs is due to its lack of calcium. But that would scarcely explain the prompt appearance of tetany after a meat meal nor the injurious action of extract of beef (50). But it is not necessary to invoke the action of a nitrogenous toxin of unknown nature. Both Salvesen and the protagonists of the toxin theory seem to have overlooked the high phosphate content of meat. This phosphate must play a part. It may be that it causes an increased excretion of calcium; it may be that it is absorbed and shifts the series of equilibria  $\text{Ca}''$  (certain tissues)  $\rightleftharpoons \text{Ca}''$  (blood)  $\rightleftharpoons \text{Ca}_3(\text{PO}_4)_2$  (other tissues) to the right. It may be that it causes either, or both, depending upon the store of available calcium in the body and upon the degree of saturation of the calcium phosphate depots.

The beneficial effects of lactose-containing diets (51, 52, 53) are probably due, in part, to the calcium such diets almost inevitably contain and, in part, to the fact that such diets contain very little phosphorus so that all of the calcium they contain is available for maintaining the calcium content of the essential tissues. In addition, the products of the fermentation of lactose, by producing a slightly more acid reaction of the blood, may stimulate the excretion of phosphate by the kidneys and thus make more calcium available by displacing the equilibria:  $\text{Ca}''$  (essential tissues)  $\rightleftharpoons \text{Ca}'' + ''\text{PO}_4$  (blood)  $\rightleftharpoons \text{Ca}_3(\text{PO}_4)_2$  (other tissues) to the left. In Inouye's experiments (53), the acidity developed in the intestine from the lactose or galactose fed may well have been sufficient to make the calcium of the bone ash in the food more available for absorption (Irving and Ferguson (54)). Inouye found that the sera of normal dogs fed on the lactose-containing diets contained more calcium than did the sera of the same animals, on other diets, also containing bone ash.

The favorable effects of the administration of large amounts of Ringer's solution (Luckhardt and Rosenbloom (55)) are due, in part at least, to the calcium thus provided. Calcium-free

Ringer's solution is less efficacious (Luckhardt and Rosenbloom (56)) and, in all probability, the beneficial effect of the diuresis it causes is due, not to the washing out of an unknown nitrogenous toxin, for whose existence no satisfactory evidence has even been produced (57) but to an increased excretion of phosphate.

The action of hydrochloric acid in relieving the tetany of parathyroidectomized dogs (Wilson, Stearns, and Janney (58)) is probably due to the removal of phosphate. This has not been demonstrated for such animals but an increased excretion of phosphate in children with tetany, following the therapeutic administration of hydrochloric acid or ammonium chloride has been reported by Scheer (59), György (60), and Anderson and Graham (61).

That estrus, pregnancy, and lactation may cause tetany to develop in dogs that had previously been kept free from tetany is readily understood in terms of the theory here advanced. Pregnancy and lactation are known to change the usual equilibria controlling the calcium metabolism and it is not unlikely that estrus does the same. In all three conditions, the tetany can be controlled by the addition of calcium salts to the food (48, 49, 52).

It is curious that healing rickets should frequently be associated with tetany. Hess and Lundagen (62) have suggested that "the seasonal increase of phosphate, beginning in April and ascending still higher in May, tends in some cases to the development of tetany late in the spring." Karger (63) has called attention to the frequent occurrence of tetany in children treated with ultra-violet radiation and has ascribed the increased incidence of tetany in April to the increased amount of solar radiation. It would seem as if, in such cases, the processes making for calcium deposition were stimulated to so great an extent as to overpower the parathyroid, or calcium-dissolving, function. The hyperplasia of the parathyroids observed by Grant and Gates (64) in irradiated rabbits may have been due to an attempted compensation for an increased tendency to calcium deposition brought about by the irradiation.

It may seem to some that in this discussion of the work of others there has been too much of what is merely probable and that more direct evidence is desirable. That is freely granted. But it is quite impossible to repeat all these experiments and to

determine the changes in the phosphorus and calcium metabolism. The quotation with which this paper begins was followed, in the original, by "No theory of the causation of tetany can be considered adequate if it fails to take both of these into consideration." Now, we would add: *It is impossible to interpret satisfactorily the results of any experiments upon the function of the parathyroid glands unless the changes in phosphorus and calcium content of blood, urine, and feces brought about by the experimental procedure are known.*

The hypothesis presented in this paper will probably require modification, it may even be proved to be totally unsatisfactory but there can be no doubt but that the tetany of parathyroidectomized dogs is due to the disturbance in the calcium and phosphorus metabolism, whatever may be the precise mechanism by which that disturbance is brought about.

#### SUMMARY.

1. The administration of calcium salts to thyroparathyroidectomized dogs led, at first, to a deposition of a large part of this calcium in the tissues, chiefly as calcium phosphate. Later, calcium equilibrium was attained.

2. A parathyroidectomized dog receiving a liberal supply of calcium had a normal concentration of calcium in the serum and was as resistant to the administration of sodium phosphate (50 mg. of phosphorus per kilo of body weight) as were normal dogs. Later, when the supply of calcium was diminished, the concentration of calcium in the serum was much less and the injection of the same amount of phosphate was followed by tetany.

3. It is suggested that the calcium content of the plasma is, normally, maintained at a constant level by an equilibrium between inorganic calcium and an organic compound of calcium. Resemblances between this organic compound and calcium citrate are indicated, but the substances are not identical.

4. It is suggested that the parathyroid hormone is necessary to the preparation of this organic constituent. The implications of this hypothesis are discussed.

The nature of this calcium-dissolving constituent is being investigated.

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## THE FATE OF CREATINE WHEN ADMINISTERED TO MAN.

By ALFRED CHANUTIN.

WITH THE ASSISTANCE OF LOREN P. GUY.

*(From the Laboratory of Physiological Chemistry, University of Virginia, University.)*

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The fate of creatine in the organism has been the subject of many investigations. A recent review of the literature on this subject discloses conflicting ideas concerning creatine-creatinine metabolism. The apparent inconsistencies may be ascribed to several factors, the most outstanding being the varying quantities of creatine administered, usually quite small, together with the fact that administration of the substance was not continued over a sufficiently long period. In most cases the difficulty and expense of isolating pure creatine limited the amount which could be used for experimentation.

It is possible to omit much of the literature since one may refer to the excellent review on this subject by Hunter (1). In his classic paper Folin (2) concluded that creatine and creatinine were independent of one another in metabolism, and that the former was utilized as a food. However, the results of later investigations, particularly those of Rose and Dimmitt (3) and Benedict and Osterberg (4), have shown that ingestion of creatine may lead to an increased output of creatinine in the urine. This evidence coupled with the fact that the creatinine found in muscular tissue is apparently derived from the "creatine complex" by the action of enzymes, leads one to believe that urinary creatinine must depend upon creatine as its precursor.

The possibility of creatine acting as an anabolite has been shown by Benedict and Osterberg (4). Dogs that were fed on a basal diet to which creatine was added not only gained weight but showed a marked positive nitrogen balance. The evidence obtained in these experiments seems to indicate that creatine may serve as a food, which would confirm the idea advanced by Folin (2) in 1906.

In this investigation, we carried out feeding experiments with creatine on man. The results obtained show definitely that creatine and creatinine are not independent of one another in the body. Furthermore, these experiments demonstrate that a major portion of the creatine retained by the tissues may be converted to creatinine. Proof is given that creatine may spare protein.

#### EXPERIMENTAL PROCEDURE.

The writers served as the subjects for the present experiments, subsisting upon a creatine-free diet for 6 and 8 weeks respectively. The diet, consisting of shredded wheat, bananas, bread, butter, strawberry preserves, sugar, and milk, represented an intake of approximately 3400 calories. The diet was adhered to strictly with respect to its composition. During the experiment, daily exercise was part of the regular routine. The type of exercise indulged in was not strenuous and varied from day to day.

It was found that the nausea induced by the bitter taste of the creatine could best be avoided by dissolving it in hot weak tea. The creatine was taken about 2 hours after the noon meal. By recrystallizing commercial creatine twice, the resulting product was found to be pure upon analysis.

The urines collected at the end of 24 hours were diluted to the same volume each day with a few exceptions. Total nitrogen was estimated by the macro Kjeldahl-Gunning method and creatinine by the Folin method. Creatine was determined by the autoclave method of Folin. Creatinine zinc chloride was used as a standard for the creatine and creatinine determinations. Ammonia nitrogen was estimated by the procedure of Van Slyke and Cullen.

In order to be certain that the body was in nitrogen equilibrium the experimental diet was begun 2 weeks before the first sample of urine was collected. After a satisfactory control period during which the urinary constituents studied were fairly constant, the administration of creatine was begun.

The detailed results of the experiments are recorded in Tables I and II. The experimental periods lasted 29 and 44 days during which time 250 and 340 gm. of creatine, respectively, were ingested. The analyses for July 12 cannot be considered because

of a mistake made in taking unknown quantities of creatine on the previous day. These data have been omitted from the averages obtained for this period.

### *Results.*

During the first few days of creatine feeding its retention by the body is striking. A gradual decrease in retention may be noted, however, as the experiment proceeds. On the other hand, the extra creatinine eliminated rises slowly towards a maximum level. It is interesting to note that the smallest creatine and extra creatinine output during the entire experiment is obtained after the 1st day of creatine feeding. If creatinine is to be considered as the end-product of creatine metabolism we must assume that creatine may be stored without any appreciable breakdown.

Apparently the ability of the tissues to store creatine should reach its maximum after the daily ingestion of 10 gm. of creatine over a period lasting more than a week. At this point Subject A. C. (Table I) doubled the daily dose of creatine fed. The figures for the extra creatinine eliminated and the creatine retained both show marked increases. In fact the output of extra creatinine on the 2nd day of this period shows an increase of about 160 per cent over the control period. We believe this figure represents the largest percentage creatinine output due to creatine feeding recorded in the literature. The unexpected increased retention of creatine indicates that the creatine reservoir may be larger than has been hitherto thought possible. As the period progresses the amount of creatine retained becomes smaller.

At the end of an 8 day period during which time 160 gm. of creatine were taken, the daily dose was dropped back to 10 gm. Although this period lasted but 2 days evidence regarding creatine storage in the body is given. The excretion of creatine was greater than the intake on the 1st day.

Subject L. P. G. continued to consume 10 gm. of creatine daily over a period of 34 days. In Table II it is seen that the creatine and creatinine excretion rose slowly until a fairly even level was reached at about the end of the first period. Although the



TABLE I.  
Experiment 1. Fate of Ingested Creatine.  
Subject A. C.

The figures for creatine are expressed as creatinine.

Date.	Volume of urine.	Total N.	NH <sub>3</sub>	Total creatinine.	Preformed creatinine.	Creatine.	Creatine retained.	Extra creatinine eliminated.	Percentage of retained creatinine eliminated.	Weight.	
1895	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	kg.	
June 20										70.4	Preliminary period of 7 days.
" 29										72.0	No creatine given.
July 1	840	7.80	0.327	1.49	1.49						
" 2	850	7.77	0.271	1.52	1.52						
" 3	1,020	7.82	0.339	1.54	1.54						
" 4	730	7.41	0.315	1.55	1.55						
" 5	590	7.53	0.347	1.46	1.46						
" 6	685	7.66	0.241	1.49	1.49						
" 7	470	7.42	0.336	1.53	1.53					72.0	
		7.62	0.311	1.51	1.51						Average daily output.
July 8	690	9.04	0.378	4.44	1.73	2.71	5.91	0.22			
" 9	595	8.60	0.436	5.26	1.96	3.30	5.32	0.45			
" 10	1,090	9.57	0.554	6.12	2.14	3.98	4.64	0.63			
" 11	970	9.70	0.571	6.53	1.90	4.63	3.99	0.39			
" 12*	740	8.52	0.549	2.96	1.73	1.23					First period of creatine administration. 10 gm. of creatine (8.62 gm. creatinine) ingested daily.

July 13	715	8.82	0.420	5.84	2.22	3.62	5.00	0.71	13	Average daily output.
" 14	610	9.13	0.431	6.15	2.17	3.98	4.64	0.66		
" 15	735	9.02	0.532	6.16	2.26	3.90	4.72	0.75		
" 16	710	8.96	0.473	7.14	2.32	4.82	3.80	0.81		
" 17	650	9.46	0.448	8.11	2.32	5.79	2.83	0.81		
		9.14	0.471	6.19	2.10	4.08	4.54	0.60	74.0	Second period of creatine administration. 20 gm. of creatine (17.24 gm. creatinine) ingested daily.
July 18	1,200	12.56	0.445	16.38	3.59	12.79	4.45	2.08		
" 19	1,240	12.56	0.445	15.54	4.05	11.49	5.75	2.54		
" 20	780	12.12	0.420	16.12	3.88	12.24	5.00	2.37		
" 21	990	12.37	0.414	17.24	3.66	13.58	3.66	2.15		
" 22	860	11.80	0.381	15.87	3.46	12.41	4.83	1.95		
" 23	930	12.37	0.398	16.00	2.74	13.26	3.93	1.23		
" 24	960	12.88	0.386	16.00	2.70	13.30	3.94	1.19		
" 25	1,275	12.31	0.487	18.45	3.42	15.03	2.21	1.91		
		12.37	0.422	16.45	3.44	13.01	4.23	1.93	45	Average daily output.
July 26	940	9.99	0.429	11.66	2.32	9.32	-0.70	0.81		
" 27	670	9.48	0.429	11.17	3.02	8.15	0.47	1.51		
		9.73	0.429	11.43	2.67	8.73	-0.12	1.16		
		7.14	0.420	4.62	2.20	2.42		0.69		
July 28	490	8.03	0.408	2.80	2.12	0.68		0.61	75.2	Fourth period. No creatine ingested.
" 29	620									
		7.58	0.414	3.71	2.16	1.55		0.65		

Figures for day not averaged.

TABLE II.  
*Experiment 2. Fate of Ingested Creatine.*  
*Subject L. P. G.*

The figures for creatine are expressed as creatinine.

Date.	Volume of urine.	Total N.	NH <sub>4</sub>	Total creatinine.	Preformed creatinine.	Creatine.	Creatine retained.	Extra creatinine eliminated.	Percentage of retained creatine eliminated.	Weight.	
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	kg.	
1915											
June 20										68.5	
" 29										68.5	
July 1	670	8.69	0.476	1.66	1.66						
" 2	770	7.69	0.256	1.49	1.49						
" 3	750	8.40	0.268	1.77	1.77						
" 4	620	8.42	0.336	1.65	1.65						
" 5	510	7.91	0.282	1.60	1.60						
" 6	520	7.20	0.370	1.54	1.54						
" 7	530	7.80	0.402	1.60	1.60						
		8.01	0.341	1.61	1.61					69.5	
											Preliminary period of 7 days.
											No creatine given.
											Average daily output.
July 8	575	7.91	0.260	3.64	1.82	1.82	6.80	0.21			
" 9	550	8.37	0.380	4.32	1.85	2.47	6.15	0.24			
" 10	670	8.22	0.424	5.63	2.22	3.41	5.21	0.61			
" 11	640	9.18	0.409	5.40	1.98	3.42	5.20	0.37			
" 12*	1,810	10.41	0.437	8.42	2.22						

[illegible]

TABLE II—*Concluded.*

Date.	Volume of urine.	Total N.	NH <sub>3</sub>	Total creatinine.	Preformed creatinine.	Creatine.	Creatine retained.	Extra creatinine eliminated.	Percentage of retained creatine eliminated as creatinine.	Weight.	
	cc.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	per cent	kg	
1925											
Aug. 7	830	10.22	0.375	10.00	2.46	7.54	1.08	0.85			Fourth period of creatine administration.
" 8	690	9.86	0.386	8.86	2.34	6.52	2.10	0.73			10 gm. creatine (8.62 gm. creatinine) ingested daily.
" 9	690	9.88	0.389	9.33	2.38	6.95	1.67	0.77			
" 10	710	10.60	0.347	11.40	2.80	8.60	0.02	1.19		72.0	
" 11	540	9.74	0.342	10.72	2.72	8.00	0.62	1.11			Average daily output.
		10.06	0.368	10.06	2.54	7.52	1.09	0.93	85		Fifth period. No creatine ingested.
Aug. 12	500	9.46	0.386	3.15	2.34	0.81		0.73			
" 13	680	8.30	0.364	2.50	2.30	0.20		0.69		72.3	
		8.88	0.375	2.82	2.32	0.50		0.71			Average daily output.

\* Figures for day not averaged.

figures for creatinine elimination show slight daily variations throughout the remainder of the experiment, the averages obtained for the various periods correspond closely. On further analysis of the data it will be seen that the amount of extra creatinine eliminated is almost the same as that of creatine retained (in terms of creatinine). During the first period 14 per cent of the creatine retained was recovered as extra creatinine. The second, third, and fourth periods show a conversion of 90, 126, and 85 per cent. In Subject A. C. 45 per cent of the retained creatine is converted to creatinine during the second period. These data would lead us to conclude that a considerable portion of the creatine retained by the body may be transformed to its anhydride during the metabolic cycle under the conditions of these experiments.

The work of Fowler and Hawk (5) showed that creatine appears in the urine of normal adults after copious water drinking. Large quantities of water were drunk by Subject L. P. G. to note what effect it would have on creatine excretion. On August 1 this subject secreted 2720 cc. of urine which was accompanied by an increase over the preceding day of more than 5 gm. of creatine. An almost corresponding diuresis on the following day failed to produce this result again. No effect on creatinine excretion was noted.

In Tables III and IV the results are summarized in terms of nitrogen elimination. The figures for the nitrogen balance are significant. In estimating the nitrogen derived from ingested creatine, it is necessary to include the extra creatinine. Hence the addition of the average for the basal total nitrogen plus the nitrogen of total creatine and extra creatinine should give the theoretical total nitrogen elimination. The results show that the figures obtained for total nitrogen are much lower than would be expected. The feeding of creatine seems to act as a protein-sparer. Although the creatine retained is for the most part converted to creatinine (Subject L. P. G.), the evidence still points to a storing of nitrogen. Benedict and Osterberg (4) suggest that "creatine may cause nitrogen storage in the body far beyond that contained in creatine itself."

During the experimental period both subjects increased steadily in weight (Tables I and II). An increase of about 3 kilos in

TABLE III.  
*Results Summarized in Terms of Nitrogen Output.*  
*Subject A.C.*

The figures are for the average daily output.

Period.	Total N.	Total creatinine N.	Preformed creatinine N.	Creatine N.	Extra creatinine N.	Creatine N plus extra creatinine N eliminated.	Creatine N eliminated plus basal N.	Creatine N intake.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Preliminary (7 days).	7.62	0.56	0.56					
I (10 days).	9.14	2.29	0.78	1.51	0.22	1.73	9.35	3.71
II ( 8 " ).	12.37	6.10	1.27	4.83	0.72	5.55	13.17	7.42
III ( 2 " ).	9.73	4.24	0.99	3.24	0.43	3.67	11.29	3.71
IV ( 2 " ).	7.58	1.37	0.80	0.58	0.24	0.82	8.44	

TABLE IV.  
*Results Summarized in Terms of Nitrogen Output.*  
*Subject L. P. G.*

The figures are for the average daily output.

Period.	Total N.	Total creatinine N.	Preformed creatinine N.	Creatine N.	Extra creatinine N.	Creatine N plus extra creatinine N eliminated.	Creatine N eliminated plus basal N.	Creatine N intake.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Preliminary (7 days).	8.01	0.60	0.60					
I (10 days).	9.29	2.36	0.83	1.53	0.23	1.76	9.77	3.71
II (10 " ).	10.55	3.75	1.05	2.69	0.45	3.14	11.15	3.71
III (10 " ).	10.63	3.88	0.98	2.89	0.38	3.27	11.28	3.71
IV ( 5 " ).	10.06	3.73	0.94	2.79	0.35	3.14	11.15	3.71
V ( 2 " ).	8.88	1.05	0.86	0.19	0.26	0.45	8.46	

body weight during a short experimental period is rather unusual. This addition of weight is noted during the ingestion of the basal diet, but we are inclined to believe that much of the subsequent

increase is related in some way to creatine ingestion. These results are suggestive and might stimulate further research under more carefully controlled conditions.

#### DISCUSSION.

A discussion of the creatine-creatinine metabolism must of necessity be of somewhat hypothetical nature because of the number of unknown factors to be dealt with. In order to study this problem these experiments were made under fairly constant conditions by flooding the organism with a large excess of creatine.

The present research has shown that when creatine is given to normal adults in large doses *per os*, a definite transformation of creatine to creatinine is possible. No definite percentage transformation can be postulated since the reaction apparently depends entirely upon the equilibrium established in the "active tissue." No marked increase in extra creatinine is noted until the body has retained much of the creatine administered. Accordingly, it is not surprising that small doses, which undoubtedly can be easily retained in a creatine reservoir, would not be traced by analysis of the urine. After small doses, creatine may possibly be stored and metabolized slowly, just as fat or glycogen which has been deposited in its respective depots.

The significance of creatine in the tissues is unknown, although it is present in relatively large amounts. Its behavior in disease, muscular exercise, protein feeding, and fasting would lead one to assume that there is a close relationship with protein metabolism. These experiments suggest that creatine, in some manner, may under certain conditions, play a rôle in determining the nitrogen balance of the organism. It is unlikely that much, if any, creatine is converted to protein since extra creatinine continues to be excreted after creatine ingestion is stopped. The relationship of creatine storage to protein metabolism cannot be postulated without more definite evidence.

If creatine can be stored in the tissues we can calculate roughly the quantity retained. Of the 340 gm. of creatine taken by Subject L. P. G., it is found that 38 gm. are held by the body at the end of the experiment. Subject A. C. retained 58 gm. of creatine after taking 270 gm. The apparent discrepancy in results is due to the time in which the body was allowed to react



to the respective quantities of creatine taken. Since the subjects each weighed about 70 kilos, the creatine content of the muscles may be calculated roughly at about 115 gm. This would mean that there was increased storage in the muscles amounting to about 33 and 50 per cent. This retention does not seem improbable in the light of the experiments of Folin and Denis (6) and Myers and Fine (7), who demonstrated that muscles could hold, temporarily, at least, even larger quantities of added creatine than have been noted in these experiments.

The inability to discover the fate of small quantities of creatine is responsible for the theory that creatine is destroyed in the alimentary tract through bacterial action. Folin (2) was unable to find any trace of creatine in the feces after feeding. The experiments of Rose and Dimmitt (3) indicate that creatine is not decomposed in the alimentary tract since there is no appreciable increase in urinary urea or ammonia. The figures obtained for the conversion of retained creatine to creatinine in these experiments would lead us to assume that creatine is completely absorbed from the alimentary tract, carried to the tissues, and either stored there or rejected at once and eliminated through the kidneys.

It is a generally accepted fact that there is an active mass of tissue where creatine is built up and stored as an unstable complex form which apparently has an important rôle in the normal functioning of the protoplasmic mass. The organism under normal conditions apparently has its full quota of creatine. However, it seems that the tissues can completely store a small amount of ingested creatine during a limited period of feeding. Large amounts are only partially retained—a retention which depends entirely upon the period of administration. Metabolic studies would lead one to suggest that stored creatine may act as an anabolite in protein metabolism. We believe that an analogy may be drawn between creatine and glucose metabolism. Small quantities of both these substances are completely retained by the body after being fed. Upon increasing the dosage, both substances will appear in the urine in amounts depending entirely on the quantities ingested. Glucose is stored as the polysaccharide glycogen and it is probable that creatine may be built up in a similar manner to a polycreatine compound. Whereas

glucose is broken down to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ , the end-product of creatine metabolism is creatinine in all likelihood or some creatinine precursor. Undoubtedly both these reactions take place through the action of enzymes. Creatine may thus be stored as a reserve food material and utilized very slowly until the normal level is reached.

#### SUMMARY AND CONCLUSIONS.

1. The absorption of creatine from the alimentary tract appears to be complete. There is no evidence of its bacterial destruction in the alimentary tract.

2. The creatinine content of the urine in man increases after ingestion of large doses of creatine. Data are presented to prove that extra creatinine excretion is derived directly from the creatine fed.

3. Evidence is presented to indicate that creatine has an indirect action on nitrogen metabolism.

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## IRON IN NUTRITION.

### II. QUANTITATIVE METHODS FOR THE DETERMINATION OF IRON IN BIOLOGICAL MATERIALS.\*

By C. A. ELVEHJEM AND E. B. HART.

(From the Department of Agricultural Chemistry, University of Wisconsin,  
Madison.)

(Received for publication, October 26, 1925.)

There is no satisfactory quantitative method at present for the determination of small amounts of iron in all plant and animal materials. One of the older methods, in general use, which involves ashing and reduction of the iron to the ferrous state and then titration with potassium permanganate has two difficulties in its operation,—a suitable and effective method of reduction and the attainment of a sharp end-reaction with the permanganate. Recently Murray (1) has improved this method, particularly in respect to the reduction procedure, and apparently has obtained excellent results on material like blood where the iron content is relatively high. The Neumann method (2) involving the principle of precipitating the iron with ammonium hydroxide in a sulfuric acid solution containing zinc sulfate and sodium phosphate, with the final estimation of the iron iodometrically, has been criticized as not quantitative, there being an incomplete precipitation of the iron in this method.

The most highly recommended method is the one developed by Thomson (3). This method is recommended by the American Public Health Association in their Standard Methods for the Examination of Water and Sewage, 1923. It involves the principle of ashing; dissolving the ash in *hydrochloric acid*; adding a small amount of potassium permanganate to insure oxidation of all the iron to the ferric state; and forming the deep red color of ferric thiocyanate through the addition of potassium thio-

\* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

cyanate, with a final colorimetric determination against a standard iron solution. This method was designed as particularly applicable to small amounts of iron.

When we tried to use this method for the determination of iron in milks, cabbage, and other materials, we at once encountered difficulties. Upon addition of the potassium thiocyanate the color would immediately fade and in many cases disappear entirely. For example, the ordinary procedure with this method gave absolutely no indication of the presence of iron in cow's milk, although the older method of Neumann, which had been used by Von Bunge (4) and Abderhalden (5) indicated a content of 2 to 3 mg. of iron per kilo of liquid milk. Von Bunge also used the simpler procedure of precipitating the iron as a phosphate in the presence of ammonium acetate and then estimated the dissolved iron by titration. We believed the fading of the color was due to the presence of phosphates as there are several references in the literature (6) stating that the red color is destroyed by phosphates, borates, arsenates, and certain organic acids such as acetic, oxalic, tartaric, citric, malic, succinic, etc. Of these the only possible interfering radical remaining after ignition of a sample of milk is that of phosphorus. This color fading was readily demonstrated; the addition of small amounts of a 1 per cent solution of  $\text{Na}_2\text{HPO}_4$  caused immediate fading of a standard iron solution in which the color had been developed by thiocyanate.

The delicacy of the thiocyanate method makes it highly desirable to use if the objections mentioned above can be removed. The problem therefore was to find a method of separating the phosphorus from the iron so that the iron could be determined in a phosphorus-free solution. Of course the first possibility was to separate the phosphorus by precipitation. This was done with ammonium molybdate, but upon direct use of the filtrate for the iron determination it was found that the remaining excess of molybdate solution caused a greater fading than the original amount of phosphates present in the sample.

The next procedure followed was to precipitate the iron, leaving the phosphate behind. In order to simplify our method in these trials we used a 200 cc. solution containing 0.5 gm. of  $\text{Na}_2\text{HPO}_4$  and in most cases 0.3 mg. of iron instead of a solution

containing the ash from 100 cc. of milk. This solution would, therefore, contain about the same amount of  $P_2O_5$  as that obtained from the milk. The first method we tried for the precipitation of the iron was the basic acetate method (7). Carrying out this method very carefully no precipitate could be obtained in the presence of 0.3 mg. of Fe and not even if the amount of iron was doubled to 0.6 mg. of Fe. Therefore this method could not be used unless a very large sample of milk was taken, which would not always be practicable.

TABLE I.  
*Iron Determinations.*

Duboseq readings.	
Standard containing 0.3 mg. Fe.	Phosphate solution from which 0.3 mg. Fe was recovered with KOH.
20	20.5
20	20.3
20	20.1

TABLE II.  
*Iron Determinations.*

Duboseq readings.		
Standard containing 0.3 mg. Fe.		Phosphate solution from which 0.3 mg. Fe was recovered with KOH.
Platinum Gooch.	20	20.3
	20	20.1
	20	20.1
Asbestos Gooch.	20	19.6
	20	20.1
	20	20.3

Another possibility was the direct precipitation of the iron in an alkaline solution. Of course in such a solution the iron would be precipitated as  $FePO_4$ , but due to the small amount of iron only a minute quantity of phosphorus would come down and therefore would do no harm. Ammonium hydroxide was used, but no precipitate was obtained in the presence of such small quantities of iron. A brownish color developed but the colloidal iron would not separate even by coaxing through the

addition of electrolytes or through further evaporation of the solution. When KOH was used in place of the  $\text{NH}_4\text{OH}$  an excellent precipitate of  $\text{FePO}_4$  and  $\text{Fe}(\text{OH})_3$  was obtained which settled very nicely. Upon filtering the precipitate off and comparing the color developed upon addition of potassium thiocyanate to the dissolved precipitate, it was found that the iron could be recovered quantitatively (see Table I).

In order to make our solution more nearly like that of milk 0.5 gm. of  $\text{CaHPO}_4$  was used in the 200 cc. of solution instead of  $\text{Na}_2\text{HPO}_4$ . Even in the presence of the calcium which would mean the precipitation of some  $\text{Ca}_3(\text{PO}_4)_2$  in an alkaline solution not enough phosphate was precipitated to interfere and the iron was again recovered quantitatively (see Table II).

The method for iron determination outlined above looked very promising, but when applied to milk the increased amount of calcium contained in the milk over that contained in the artificially prepared solution we had been using brought down enough phosphorus upon addition of KOH to cause difficulties, due to fading. The possibility suggested as a remedy for this trouble was to remove the calcium as a first procedure. The best way to remove calcium is as an oxalate, but we would be adding a substance which causes color fading. However, the oxalate could be destroyed by ignition, but this would lengthen the procedure beyond practicability. The last possibility tried and which we found to be very satisfactory was to remove the phosphorus first with ammonium molybdate and then precipitate the Fe with KOH. The molybdate precipitation removed the phosphorus completely, leaving merely the excess molybdate solution, iron, calcium, and magnesium in the filtrate. Upon addition of KOH the molybdate would then be made soluble as potassium molybdate and the Fe precipitated as  $\text{Fe}(\text{OH})_3$  along with the calcium and magnesium hydroxides. These may be filtered off on either a spongy platinum Gooch or an acid-washed asbestos Gooch crucible and the iron determined colorimetrically after dissolving in HCl. After making a number of analyses on biological materials very rich in phosphorus as compared to their iron content, it is believed that the method as developed is highly satisfactory for the determination of iron in milks and other biological materials.

TABLE III.  
*Iron Determinations in Milks.*

Material.	Size of sample.	No. of sample.	Fe <i>per cent</i>	Remarks.
Whole milk powder.	5 gm.	1	0.0032	
" " "	"	2	0.0032	
" " "	"	3	0.00322	
" " "	"	4	0.00312	
" " "	"	5*	0.0050	0.1 mg. Fe added.
" " "	4 gm.	1	0.00325	
" " "	"	2	0.00312	
" " "	"	3	0.00312	
" " "	"	4	0.00325	
" " "	"	5*	0.0054	0.1 mg. Fe added.
Whole liquid milk.	50 cc.	1	0.00036	
" " "	"	2	0.00036	
" " "	"	3	0.00035	
" " "	"	4*	0.000548	0.1 mg. Fe added.
" " "	"	5*	0.00054	0.1 " " "

\* 0.1 mg. Fe was added to the stored samples by adding 1 cc. of standard iron solution before ignition. If this amount is subtracted from the actual amount found in the sample before calculating the percentage, we find that quantitative recovery is obtained. For example, Sample 3 (whole liquid milk) contained 0.00035 per cent Fe. Sample 4 contained 0.00035 per cent after deducting the added iron.

Table III gives some of the results obtained with different milks. The details of the new method are as follows:

#### *Solutions Required.*

*Standard Iron Solution.*—Dissolve 0.7 gm. of ferrous ammonium sulfate (dried to constant weight) in 100 cc. of distilled water and add 5 cc. of concentrated sulfuric acid. Warm the solution slightly and add potassium permanganate until the iron is completely oxidized. Dilute the solution to 1 liter. 1 cc. of the standard iron solution equals 0.1 mg. Fe.

A 10 per cent solution of potassium thiocyanate.

*N/5 Potassium Permanganate.*—Dissolve 6.30 gm. of the salt in distilled water and dilute to 1 liter.

*Hydrochloric Acid.*—Concentrated, free from iron.

*Nitric Acid.*—Concentrated, free from iron.



*Molybdate Solution.*—A solution of ammonium molybdate prepared in the usual manner on which a blank iron determination has been made to insure its freedom from iron.

*40 Per Cent Solution of KOH.*—Prepared to be iron-free by making a 40 per cent solution and allowing to stand several days, decanting the iron-free solution from the top.

### *Procedure.*

A sample of the material to be analyzed is weighed out so as to contain between 0.1 to 0.3 mg. of Fe. In the case of liquid milk 50 cc. are used and evaporated to dryness. The sample is then carefully ignited in an electric furnace. A platinum dish is preferable for the ignition although a previously ignited and acid-washed porcelain evaporating dish may be used successfully. The ash is taken up in about 10 cc. of  $H_2O$  and 5 cc. of concentrated  $HCl$  and allowed to stand for several hours. The residue is filtered off and the phosphorus removed from the filtrate in the usual manner, which consists of adding concentrated  $NH_4OH$  until the solution becomes cloudy, clearing up with concentrated  $HNO_3$ , and adding 10 drops of  $HNO_3$  in excess. 30 cc. of ammonium molybdate solution is added, digested on a water bath at  $65^\circ C.$  for  $\frac{1}{2}$  hour, and the yellow precipitate of ammonium phosphomolybdate filtered off. The precipitate is carefully washed with dilute  $HNO_3$  (9 cc.  $HNO_3$  in 100 cc.  $H_2O$ ) to insure the removal of the last traces of iron to the filtrate. Redigestion is not necessary as the small traces of phosphorus remaining in the filtrate will not interfere. The solution is brought almost to a boil and 40 per cent  $KOH$  (iron-free) is added until no further precipitate forms; usually about 20 cc. are required. The solution is boiled for several minutes to remove the ammonia present. The solution is allowed to cool and if the hydroxides do not settle properly a few cc. of  $KOH$  are added and heated further. The precipitate is filtered off on an asbestos Gooch crucible, which has been carefully washed with  $HCl$ , by decanting the clear liquid first and finally adding the precipitate to the Gooch crucible. The precipitate is washed with very dilute  $KOH$  solution (1 to 2 per cent). Best results are obtained if only a low pressure is maintained on the suction flask during filtering. The precipitate

is dissolved from the Gooch crucible with  $2\frac{1}{2}$  cc. of concentrated HCl, which is added in several portions (a few drops at a time) washing with water after each addition of acid. In this way the iron may be dissolved off completely and the total filtrate kept below 30 to 35 cc. The best method of handling this small amount of solution is to introduce a test-tube into the suction flask, allowing the end of the funnel to reach into the test-tube so that the solution will be caught in the test-tube instead of the suction flask. The solution in the test-tube is then washed into the original beaker and the iron determined colorimetrically by adding enough  $N/5$   $KMnO_4$  to produce a faint pinkish color (usually 1 to 2 drops) then adding 5 cc. of a 10 per cent solution of potassium thiocyanate and making up to 50 cc. volume in a volumetric flask. The color produced is compared in a Duboscq colorimeter with a standard color developed by taking 1 cc. of the standard iron solution, adding 1 to 2 drops of  $N/5$   $KMnO_4$  and 5 cc. of the 10 per cent solution of potassium thiocyanate, and making up to 50 cc. volume. The standard solution is set at 20 in the Duboscq and the unknown adjusted until the colors are equal. Since the standard contains 0.1 mg. of iron, the amount of iron in the unknown is readily calculated. If the variation in the readings of the standard and unknown is too great a smaller or larger amount of the standard should be taken as the case may be.

Under certain conditions some difficulty may be experienced in filtering the precipitate of the hydroxide on the asbestos Gooch. This may be remedied by adding a small amount of ammonium oxalate (20 cc. of  $2\frac{1}{2}$  per cent solution) to the solution before addition of the KOH. Due to the presence of the oxalate the calcium will be precipitated as the oxalate, making the precipitate more crystalline and easier to filter. Of course, in this case, after filtering, the Gooch crucible must be dried and held in a flame for a short time to remove the oxalate or it will interfere with the color development. Upon ignition the calcium oxalate is changed to  $CaCO_3$ , and if care is taken not to continue the heating long enough to form CaO it is easily dissolved in the amount of HCl we have suggested. We do not believe this scheme necessary as we have experienced no great difficulty in filtering in any of the determinations we have made. However, we have used this

departure with success and it may be found to be useful in some cases.

During the latter part of our experimental work Walker (8) published some work on the colorimetric determination of small amounts of iron, in which he pointed out that phosphates greatly interfere with the color development. As a means of overcoming this color fading, he suggested the use of  $\text{HNO}_3$  instead of  $\text{HCl}$ . The use of  $\text{HNO}_3$  would eliminate the addition of  $\text{KMnO}_4$ , because the small amount of  $\text{HNO}_2$  present would readily oxidize the iron. He did, however, introduce a small amount of  $\text{H}_2\text{O}_2$  to oxidize any of the remaining free nitrous acid, because this acid with potassium thiocyanate gives a very slight coloration.

This method (Walker's) was tried on 50 cc. samples of milk but the same difficulty was encountered. This was expected because Walker suggested that this method was not satisfactory when the sample contained more than 0.05 gm. of phosphoric acid. A 50 cc. sample of milk would contain about 0.12 gm. Therefore the only method that is satisfactory for materials high in phosphates is the method just described.

However, we were very successful applying the nitric acid method (Walker's) to material like cabbage which contains less phosphates, but too large an amount to use the Thomson method, which involves the use of  $\text{HCl}$ . For example, when applied to cabbage the Thomson method ( $\text{HCl}$  as solvent) gave results which were only about one-third of those secured with the Walker method ( $\text{HNO}_3$  as a solvent).

The following procedure was used in these determinations.

A 5 gm. sample was weighed into a porcelain evaporating dish and ignited in an electric furnace. After cooling, 10 cc. of distilled water were added and 3 cc. of concentrated  $\text{HNO}_3$ . The insoluble residue was filtered off and the filtrate was treated with 2 cc. of  $\text{H}_2\text{O}_2$ . After standing for 1 minute, 5 cc. of a 10 per cent potassium thiocyanate solution were added and diluted to 50 cc. volume. The color developed was compared with a standard solution containing 1 cc. of iron solution and treated in the same manner except 2 cc. of concentrated  $\text{HNO}_3$  were used. 3 cc. were added to the ash because some of the nitric acid was neutralized by the alkalinity of the ash.

For materials like lettuce, grains, and hay, where the iron

content is relatively high compared with the phosphates, we have used Thomson's method directly with good results.

#### SUMMARY.

In this paper three methods are discussed for the accurate determination of iron in biological materials.

1. Thomson's method which may be used for materials relatively high in iron and low in phosphorus.

2. Walker's modification which may be used for materials somewhat rich in phosphorus.

3. A new method which may be used for materials relatively low in iron and quite high in phosphorus, such as milk.

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## THE QUESTION OF THE INTERACTION OF INSULIN, MUSCLE TISSUE, AND GLUCOSE.

By A. D. BARBOUR.

(From the Department of Biochemistry, University of Toronto, Toronto,  
Canada.)

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The discovery of a reaction *in vitro*, characteristic of insulin and furnishing a possible basis for its chemical assay, would be of the utmost practical as well as theoretical importance. Such a reaction was recently reported by Lundsgaard and Holbøll (1). These investigators found that when insulin was added to glucose in the presence of freshly excised muscle tissue at 37°C. the optical rotation of the glucose became smaller without any corresponding change in its reducing power. The change was observed by dialysing samples of the mixture in collodion tubes, and comparing the reducing and rotatory powers of the dialysates. The rotatory power was always found to be less than that demanded by the concentration of glucose as determined by reduction. The maximum effect was observed in glucose solutions of 1 to 2 per cent containing from 7.5 to 2.5 gm. of muscle tissue and from 5 to 25 clinical units of insulin per 100 cc. The greatest change reported was 44 per cent. Later papers (2) have further extended these observations.

The work of Lundsgaard and Holbøll was supported to some extent by earlier observations of Winter and Smith (3), who found that sugar in the blood of a normal animal, which is undoubtedly under the influence of insulin, possesses a rotatory power lower than that required by its copper reduction value. Winter and Smith assumed that the discrepancy was due to the conversion of part of the sugar into the unstable  $\gamma$ -glucose before utilization in the body. In diabetic blood the reducing and rotatory powers were found to agree. The experiments of these authors were criticized by Hewitt (4), but the polarimetric readings obtained

were confirmed by Eadie (5). The latter thought it unnecessary to assume a formation of  $\gamma$ -glucose, but considered the discrepancy to be due to a disturbance in the normal equilibrium between  $\alpha$ - and  $\beta$ -glucose.

An experiment has also been reported by Slosse (6), in which a loss in the rotatory power of a glucose solution, to which insulin had been added, was observed after 24 hours' incubation at 37°C. Lundsgaard and Holbøll have failed to confirm this.

#### EXPERIMENTAL.

In view of the great importance of the results of Lundsgaard and Holbøll, it was considered advisable to repeat their work, and if possible to extend the observations to include other tissues than muscle. The repetition was carried out under those conditions which yielded to Lundsgaard and Holbøll their most definite results.

For dialysis of the incubation mixtures we used collodion tubes, demonstrated by preliminary tests to be quite impermeable to protein, while readily allowing the passage of glucose. The dialysis of a glucose solution reached equilibrium in 2 hours at room temperature.

Polarimetric observations were made in a Hilger polarimeter, using 200 mm. tubes. The illumination was the green line of the spectrum of mercury vapour (wave-length 546.1) which is recommended as a standard for polarimetric observations by the United States Bureau of Standards (7). This gives for glucose  $[\alpha]_{546.1}^{20^\circ} = 62.03^\circ$ . No difficulty was experienced in obtaining readings agreeing within  $0.02^\circ$ . The readings accepted were always averages of not less than six separate readings by each of two independent observers.

The glucose used was recrystallised twice from glacial acetic acid by the method of Hudson and Dale (8). Its rotatory power was frequently checked during the course of the experiments, and it always gave, within the ordinary limits, the theoretical value.

The reducing power of the solutions was determined by the method of Shaffer and Hartmann (9). As the original table of these authors was found to be slightly inaccurate under the

conditions used, a new sugar table was constructed, using the purified sugar described above. When this table was employed, the reducing and rotatory powers of sugar solutions made up from time to time always agreed within the limits of experimental error.

The muscle tissue used was rapidly excised from freshly killed animals and minced in a sterilized meat chopper. The insulin used was Insulin—Toronto, No. 305, containing 10 clinical units per cc.

Preliminary experiments showed that on dialysing insulin alone, muscle tissue alone, and mixtures of muscle tissue and insulin, there was no measurable reducing substance in the dialysate, and no rotatory power exceeding  $0.02^\circ$ .

The protocol of one typical experiment is given below in full.

Two incubation mixtures, A and B, were prepared with the following composition: 10 gm. of rabbit muscle tissue (ground), 20 units of Insulin—Toronto, No. 305, 25 cc. of 3 per cent glucose solution, 0.9 per cent NaCl solution to 50 cc. The two identical mixtures were incubated separately for 2 hours at  $37^\circ\text{C}$ . The pH of each after incubation was 6.4. Two 10 cc. samples of each mixture were then dialysed for 2 hours at room temperature against 20 cc. of 0.9 per cent NaCl. The two dialysates of A were combined, as were those of B. Polarimetric readings were then taken of each of the mixed dialysates, of the original 3 per cent glucose solution, and of the original solution diluted to the same degree—1 in 6—as would be expected of the sugar in the dialysates. All the readings taken, with their averages and the glucose concentrations calculated from these, are recorded in Table I. The glucose concentrations are calculated from the formula

$$[\alpha] = 62.03 = \frac{100 a}{l c}$$

in which  $a$  is the observed rotation,  $l$  is the length of the tube in dm., and  $c$  is the weight in gm. of glucose in 100 cc. of the solution. The theoretical concentration of the diluted solution and of the dialysates is 0.50 per cent.

For the determination of reducing power the original glucose solution was diluted 200 times, each dialysate 40 times. The



determination in each case was made in triplicate. The results are shown in detail in Table II.

TABLE I.  
*Polarimeter Observations.*

	Original 3 per cent sugar solution.	Original sugar solution diluted 1:6.	Dialysate A.	Dialysate B.
Individual readings.	180.27°	177.20°	177.19°	177.22°
	180.27°	177.20°	177.19°	177.22°
	180.27°	177.21°	177.19°	177.22°
	180.29°	177.21°	177.21°	177.22°
	180.29°	177.20°	177.21°	177.23°
	180.27°	177.19°	177.21°	177.21°
	180.30°	177.19°	177.20°	177.22°
	180.31°	177.21°	177.22°	177.22°
	180.29°	177.20°	177.22°	177.22°
	180.29°	177.19°	177.21°	177.22°
	180.29°	177.20°	177.21°	177.22°
	180.30°	177.19°	177.21°	177.23°
Average.....	180.29°	177.20°	177.21°	177.22°
Zero point..	176.57°	176.57°	176.57°	176.57°
Observed rotation.	3.72°	0.63°	0.64°	0.65°
Calculated per cent of glucose..	3.00	0.51	0.52	0.52

TABLE II.  
*Determinations of Reducing Power.*

		Original glucose solu- tion, 3 per cent. Diluted 1:200.	Dialysate A. Diluted 1:40.	Dialysate B. Diluted 1:40.
Cc. N/200 thiosulfate.....	a	15.15	16.40	16.30
	b	15.25	16.15	16.30
	c	15.20	16.40	16.35
Average.....		15.20	16.30	16.30
Blank on reagents...		19.80	19.85	19.85
Difference.....		4.60	3.55	3.55
Per cent glucose (from tables)....		0.0150	0.0123	0.0123
“ “ “ in undiluted solu- tion.....		3.00	0.49	0.49

The results exhibited show (1) that there was no loss of glucose during the experiment and (2) that there was no appreciable

change in the relation between the rotatory and the reducing power of the sugar. If the optical activity had altered at all, it was in the direction of increase rather than decrease; but the observed difference certainly did not exceed the experimental error.

Other experiments similar to the above were carried out not only with rabbit muscle but also with muscle tissue from the rat, the dog, and finally, since it was the animal used by Lundsgaard and Holbøll themselves, the guinea pig. Experiments were also made using phosphate buffers at  $\text{pH} = 7.0$  and  $7.5$ . In every case, without exception, the polarimetric results agreed closely with those obtained by reduction. It would serve no purpose to reproduce the numerous protocols. The only point that calls for separate mention is that dialysis from the buffered solutions was rather slower than from the others.

The insulin solutions used contained a trace of tricresol. To make sure that this did not affect the reaction an experiment was performed with a preparation of insulin hydrochloride free from preservatives. The results were the same as in the previous experiments.

An experiment was also carried out, such as is described by Slosse, with 20 units of insulin in 100 cc. of 5 per cent glucose solution. No change in the rotatory or reducing power of the sugar was observed after 24 hours incubation at  $37^{\circ}\text{C}$ .

#### CONCLUSIONS.

1. In a series of experiments on mixtures of glucose and insulin in contact with rat, rabbit, dog, and guinea pig muscle tissue, it has been impossible to detect any change in the rotatory or reducing power of the sugar after 2 hours incubation at  $37^{\circ}\text{C}$ ., or any discrepancy between the concentrations of sugar obtained by determinations of the rotatory and reducing power.

2. In a mixture of insulin and glucose alone, it was found to be impossible to detect any change in the rotatory or reducing power of the sugar after 24 hours incubation at  $37^{\circ}\text{C}$ .

The author's thanks are due especially to Mr. M. D. Orr of the laboratory of the Insulin Committee of this University for his invaluable assistance and advice during the course of this work.

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## THE QUANTITATIVE DETERMINATION OF DIHYDROXY- ACETONE.\*

By WALTER R. CAMPBELL.

*(From the Department of Medicine, University of Toronto, and the Toronto General Hospital, Toronto, Canada.)*

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The question of the availability of the trioses as sources of energy and as antiketogenic agents in the animal body has been reopened by the recent publications of Isaac and Adler (1) and Rabinowitch (2) on dihydroxyacetone ( $\text{CH}_2\text{OH}\cdot\text{CO}\cdot\text{CH}_2\text{OH}$ ). A simple method of quantitative estimation of the substance in pure solution, in the presence of glucose, and in blood, has so far been lacking. Since the methods used have not distinguished qualitatively or quantitatively between the dihydroxyacetone and glucose, there remains much uncertainty as to the actual relationships of these sugars when introduced into the body; and as a knowledge of these relationships seemed essential to a study of the metabolism of dihydroxyacetone, the method hereafter described was developed to meet these requirements.

Essentially the method is an adaptation of part of the Folin-Wu (3) method for the estimation of sugar in blood. As is well known, this method consists in the reduction of cupric salts by substances split from the hexose molecule under the action of boiling alkali with subsequent reduction of a molybdenum compound in phosphoric acid solution by the cuprous oxide produced. The process yields a blue solution which is compared colorimetrically with standard colors produced from known amounts of glucose treated in a similar fashion. In the Folin-Wu process, dihydroxyacetone yields a similar color but decidedly less intense

\* The dihydroxyacetone used in the early part of these investigations was supplied gratis by the Mallinkrodt Chemical Company. Further supplies were made available through the John D. Rockefeller, Jr., grant to the Diabetic Clinic of the Toronto General Hospital.

than that obtained from an equal weight of pure glucose. Rabinowitch gives the reduction of alkaline picrates by dihydroxyacetone as 1.53 times that of glucose. For the estimation of dihydroxyacetone in pure solution these methods might be employed. They do not, however, serve to differentiate dihydroxyacetone from other sugars, such as glucose, and are, therefore, of little value in determining the amount of the substance present in the blood. As the quantity is relatively small, estimation by using cold alkaline copper solutions is rather inaccurate owing to the reoxidation of the copper in the time required to complete the reaction. It was found, however, that dihydroxyacetone reduces boiling acid phosphate molybdate solutions<sup>1</sup> directly, while the reduction from an equal quantity of pure glucose is about  $\frac{1}{10}$  as great. For practical purposes this difference is quite sufficient. The amount of glucose ordinarily present falls within colorimetric and volumetric error and from this observation the following methods have been worked out, using the revised phosphate molybdate solution suggested by Folin and Wu (3) (see Solutions).

#### *Colorimetric Method.*

After suitable dilution of the fluid containing dihydroxyacetone, 2 cc. of the diluted solution are mixed with 2 cc. of the phosphate molybdate solution in a Folin-Wu blood sugar tube and boiled in a water bath for 15 minutes. The tube is then cooled in running water. The contents of the tube are diluted to 25 cc. and compared in a colorimeter with the color developed from a suitable standard solution of dihydroxyacetone similarly treated. Standard solutions containing 0.5, 0.1, and 0.2 mg. of dihydroxyacetone per cc. furnish suitable standard colors. Standard solutions of glucose are not quite satisfactory owing to a slight difference in the quality of the colors.

*Comment.*—The unknown should not be less than three-fourths, nor more than one and one-half times the strength of the standard,

<sup>1</sup>Miller and Taylor (Miller, C. W., and Taylor, A. E., *J. Biol. Chem.*, 1914, xvii, 531) in a paper unfortunately overlooked, list dihydroxyacetone among a number of other substances which reduce ammonium molybdate in sulfuric acid solution. They state the mixture must be free from phosphoric acid and do not apply the reaction to the differential or quantitative determination of dihydroxyacetone.

as the colors do not exactly match if these limits are exceeded. Indeed it is more satisfactory, if the colorimeter is to be used, for each individual to prepare a graph, as used by Mellanby (4) and by Hunter and Campbell (5) for creatinine estimation, by actual test of dihydroxyacetone solutions of varying strengths against each other. Under these conditions the same colorimeter must always be used and care taken that it is always in exactly the same position with reference to the light source. Errors more than 25 per cent as great as the standard are found when sufficient care is not taken to observe these points. When solutions containing 0.05 and 0.2 mg. per cc. are compared with a standard containing 0.1 mg. per cc. and plunger set at 20 mm. from the bottom of the cup, the readings obtained are 44 mm. and 8 mm. respectively.

As shown later, dihydroxyacetone reduces the Folin-Wu alkaline copper tartrate reagent 0.79 times as much as an equal weight of glucose. When solutions of dihydroxyacetone containing glucose in quantity are being examined, a slight correction may be required since glucose causes reduction of the phosphate molybdate reagent  $\frac{1}{179}$  as great as dihydroxyacetone. The correction is obtained by determining the total glucose ( $M$ ) by the regular Folin-Wu procedure, the total dihydroxyacetone ( $N$ ) by the new method, and applying the equation given below:

$$Q = \frac{N - M}{179} \quad P = \frac{180 M - 0.79 N}{179}$$

when  $P$  and  $Q$  are respectively the glucose and the dihydroxyacetone content of the mixture. This is applicable only in pure solutions, where glucose is the only interfering factor.

When blood is being examined the tungstic acid filtrate as prepared by Folin and Wu is used. To 2 cc. of oxalated blood 14 cc. of water, 2 cc. of 10 per cent sodium tungstate, and 2 cc. of  $\frac{2}{3}$  normal  $H_2SO_4$  are added in the order given, mixing after each addition, and well shaken and filtered after standing 10 minutes. The filtrate is used undiluted in the method as described above, and a correction applied for the glucose and other reducing substances in the filtrate. When a standard set at 20 mm. is used, the amount of the unknown is read off on the graph and 0.05 mg. is subtracted. This purely empirical correction allows for glucose and other reducing substances in the filtrate.

*Volumetric Method.*

The second, or volumetric, method of determining the reduction caused by the dihydroxyacetone is, in my opinion, preferable to the colorimetric method. The initial steps are identical with the colorimetric method; namely, 2 cc. of the suitably diluted solution are boiled 15 minutes with an equal quantity of the acid phosphate molybdate solution and then cooled. The further procedure consists in reoxidizing the undiluted blue solution in the cold with 0.01 N  $\text{KMnO}_4$  solution from a recalibrated burette graduated in  $\frac{1}{10}$  cc. 1.14 cc. of 0.01 N  $\text{KMnO}_4$  equal  $\frac{1}{8}$  mg. of dihydroxyacetone itself; or, using a Folin-Wu blood filtrate, 1.14 cc. of the permanganate solution equal 1 mg. of dihydroxyacetone per cc. of original blood.

*Comment.*—In carrying out this titration the permanganate solution is added slowly drop by drop, with shaking, to the cold blue solution until all blue color just disappears, leaving a colorless water-clear solution. Titrating to the first pink tinge is unnecessary as the disappearance of the blue color furnishes a satisfactory end-point. When the quantities of dihydroxyacetone are large, 1 cc. of 4 N  $\text{H}_2\text{SO}_4$  may be added to dissolve the oxide of manganese formed. In the earlier part of the work all reductions of the phosphate molybdate reagent were carried out in Folin-Wu blood sugar tubes. It was found, however, that, when cooled promptly in running water, after the 15 minutes boiling, no material difference is found in the results obtained by comparative tests, using straight  $\frac{3}{4}$  inch test-tubes with the Folin-Wu sugar tubes. As the titrations can be carried out directly in the ordinary test-tubes, these have been adopted for routine work. A graduation mark on the tube at 25 cc. makes it available for colorimetric estimation if this latter method is preferred.

As previously noted, a correction is necessary in the colorimetric method for dihydroxyacetone in blood because of glucose and other reducing substances in the blood filtrate. The fact that slight reduction of the phosphate molybdate reagent with color production occurs when it is boiled with water alone makes it necessary to introduce an additional correction in the volumetric method. This correction varies slightly with chemicals obtained from different sources and is preferably established for each new lot of phosphate molybdate reagent by boiling 2 cc. of the reagent and 2 cc.

of water in a water bath for 15 minutes, cooling, and titrating with the standard permanganate solution. Seldom is this correction more than 0.06 cc. of 0.01 N  $\text{KMnO}_4$ . When Folin-Wu tungstic acid filtrates are examined the correction to be subtracted for other reducing substances present is 0.06 cc. The range for some hundreds of bloods (sugar content 0.06 to 0.4 per cent) is 0.04 cc. to 0.10 cc. 0.01 N  $\text{KMnO}_4$ . Very few bloods differ much from the average value, 0.06 cc.

#### EXPERIMENTAL.

*Reduction of Copper by Dihydroxyacetone.*—It is well known that reduction of the Folin-Wu alkaline copper tartrate solution by glucose is incomplete at the end of 6 minutes boiling. If reliable results are to be obtained by the colorimetric method it is necessary that both standard and unknown be subjected to exactly the same conditions of heating for exactly the same time. This has been found to be true for dihydroxyacetone as well. Table I shows that the reduction of copper by dihydroxyacetone proceeds at a different rate from the reduction due to glucose. At the end of 6 minutes the dihydroxyacetone has reduced but 79 per cent as much as the glucose; the reduction becomes maximal at 12 to 15 minutes though still less than that due to glucose boiled 6 minutes, and the glucose continues to reduce the copper after the 6 minute period of boiling. As previously noted, there is a slight difference in the quality of the two colors, which makes accurate comparison somewhat difficult.

*Molybdenum Reduction by Dihydroxyacetone.*—The boiling time required in the direct reduction of the phosphate molybdate reagent by dihydroxyacetone has been placed at 15 minutes rather than the 6 minutes advised by Folin and Wu for the reduction of alkaline copper tartrate solution. Several sets of experiments done both by the colorimetric and volumetric methods have shown that, while the reduction begins promptly on immersion of the tubes in the boiling water bath, it is not complete until 10 minutes have elapsed. Thereafter a very slight increase in the reduction may take place due to changes in the phosphate molybdate reagent itself. As the increase in reduction due to this factor from the 10th to the 20th minute is negligible, a 15 minute period of boiling has been selected in order to cover any possible delays in the reduction



reaction due to variations in concentration of the dihydroxyacetone, incomplete mixing, *et cetera*. At the end of this time the tube should be promptly cooled and then the color remains constant for a considerable time. Table II shows the reduction of the phosphate molybdate reagent by equal amounts of dihydroxyacetone, with various periods of boiling in terms of the volume of 0.01 N permanganate solution used.

The concentration of glucose in the reacting mixture determines the amount of cuprous oxide produced, but the result is only

TABLE I.

*Relative Color Value of Equal Quantities of Glucose and Dihydroxyacetone When Boiled with Folin-Wu Alkaline Copper Tartrate Reagent.*

Dihydroxy- acetone boiling period.	Dihydroxyacetone compared with:			
	Standard I.* Colorimeter reading.		Standard II.† Colorimeter reading.	
<i>min.</i>	<i>mm.</i>	<i>per cent</i>	<i>mm.</i>	<i>per cent</i>
3	28.5	70	‡	
4	25.5	78	8.0	250
5	25.3	79	20.0	100
6	25.3	79	25.3	79
7	23.5	85	25.1	80
8	23.0	87	25.3	79
9	23.0	87	25.5	78
10	22.5	89	26.5	75
12	21.5	93	27.4	73
15	21.3	94	27.5	73

\* Standard I, glucose boiled 6 minutes, set at 20 mm.

† Standard II, glucose boiled same time as dihydroxyacetone, set at 20 mm.

‡ Standard too faintly colored for comparison.

roughly proportional to the glucose present. For accurate work, tables of correction are necessary to determine the amount of sugar originally present. Within the limits of concentration necessary the reduction of the acid phosphate molybdate reagent by dihydroxyacetone is directly proportional to the amount of the triose present. This is illustrated in Table III in terms of the amount of permanganate used to reoxidize the reagent.

*Dihydroxyacetone in Blood.*—Some interest may attach to the behavior of dihydroxyacetone when administered by mouth to

normal individuals. 100 gm. of this substance were given in 200 cc. of water to several individuals known to be normal, at least as far as carbohydrate metabolism is concerned. These tests were controlled by giving the subject 100 gm. of glucose 2 days before or

TABLE II.

*Effect of Time of Boiling on the Reduction of the Phosphate Molybdate Reagent by Dihydroxyacetone.*

Boiling period.	0.01 N $\text{KMnO}_4$ used.*
min.	cc.
6	1.02
8	1.05
10	1.12
12	1.12
15	1.14
20	1.14

\* Corrected for blank.

TABLE III.

*Showing Direct Proportionality between Dihydroxyacetone Present and Permanganate Used.*

Dihydroxyacetone present.	0.01 N $\text{KMnO}_4$ used.*	Dihydroxyacetone found.	Error.
mg. per cc.	cc.	mg. per cc.	per cent
0.020	0.24	0.020	0
0.025	0.29	0.025	0
0.040	0.47	0.040	0
0.050	0.60	0.051	+2
0.075	0.89	0.075	0
0.10	1.18†	0.10	
0.15	1.79	0.151	+0.66
0.20	2.37	0.20	0
0.30	3.58	0.30	0
0.40	4.72	0.40	0

\* Corrected for blank.

† Used as standard of comparison.

2 days after the dihydroxyacetone and performing a blood and urinary sugar tolerance test in the usual way. None of these patients on either day excreted abnormal amounts of sugar or dihydroxyacetone in the urine. The blood glucose tolerance test in four cases is given in Table IV. In Table V are given the total

sugar, Folin-Wu method; the titration value, new method, in cc. of 0.01 N  $\text{KMnO}_4$  (corrected for blank); the blood glucose value; and the dihydroxyacetone content of the blood. While this pa-

TABLE IV.  
*Blood Sugar Tolerance Tests—100 Gm. Glucose.*

Subject.	Fasting.	30 min.	1 hr.	2 hrs.	3 hrs.
Pn	0.91	1.09	1.20	1.38	0.82
F	0.87	1.27	1.04	0.71	0.68
Pk	0.82	1.11	0.80	0.80	0.75
D	0.90	1.08	1.19	1.12	0.71

Sugar expressed in gm. per liter of blood.

TABLE V.  
*Blood Examinations Following Administration of 100 Gm. of Dihydroxyacetone.*

Subject.	Test.	Fasting.	30 min.	1 hr.	2 hrs.	3 hrs.
Pn	Total sugar.	0.92	1.64	1.07	0.63	0.81
	$\text{KMnO}_4$ cc.	0	0.80	0.25	0	0
	"Glucose."	0.92	1.10	0.90	0.63	0.81
	Dihydroxyacetone.	0	0.70	0.22	0	0
F	Total sugar.	0.95	1.34	0.88	0.84	1.02
	$\text{KMnO}_4$ cc.	0	0.33	0.21	0	0
	"Glucose."	0.95	1.11	0.64	0.84	1.02
	Dihydroxyacetone.	0	0.29	0.18	0	0
Pk	Total sugar.	0.93	1.31	1.49	0.89	0.60
	$\text{KMnO}_4$ cc.	0	0.64	0.70	0.1	0
	"Glucose."	0.93	0.87	1.02	0.82	0.60
	Dihydroxyacetone.	0	0.56	0.61	0.09	0
D	Total sugar.	0.87	1.04	0.56	0.59	0.78
	$\text{KMnO}_4$ cc.	0	0.47	0.18	0	0
	"Glucose."	0.87	0.72	0.44	0.59	0.78
	Dihydroxyacetone.	0	0.41	0.16	0	0

Sugar, glucose, and dihydroxyacetone in gm. per liter of blood.

per was being prepared for publication a paper by Rabinowitch (6) appeared showing the effects on the blood sugar of the administration of dihydroxyacetone. By the methods which he used 'he

failed to detect any of this substance in the blood. These methods we have found are not sufficiently delicate. We had also applied them to the blood filtrates, Table V, with negative results. Further comment on these results is reserved for a further communication. Incidentally, however, the discovery of this triose in quite appreciable amounts in blood lends some support to the view that other carbohydrates are not necessarily totally converted into glucose before absorption into the blood stream.

A certain theoretical interest attaches to the question whether dihydroxyacetone is normally present in blood since it has been thought to be produced as an intermediary compound in the metabolism of the carbohydrates. Having regard to the two blank determinations on reagents and on blood filtrates, the amount present in human blood (taken fasting and all stages of digestion) cannot exceed 0.005 of 1 per cent. It seems more than likely that the undetermined reducing agent responsible for this slight reduction of the phosphate molybdate reagent will be discovered among the large number of reducing substances—levulose, polyphenols, creatine, *et cetera*—known to be present in small amounts in the blood. This may not be true in all species, however, as the blank determination on rabbit blood is sometimes double that of human blood. No explanation for this discrepancy is advanced, but it seems apparent that the substance is present only in the most minute concentration in the blood stream, if at all, and, while some doubt exists as to whether the blood accurately reflects tissue metabolism, presumably little exists in the tissue cells.

The above method of determining dihydroxyacetone in pure solutions, in the presence of glucose, and in blood, is obviously not directly applicable to urine which contains in appreciable quantity numerous other substances capable of reducing the phosphate molybdate reagent. Its development, however, has permitted the investigation of several interesting phases in the metabolism of the triose, the results of which we hope to communicate in the near future.

#### *Solutions.*

*Dihydroxyacetone.*—A 1 per cent solution is prepared by dissolving 1 gm. of dihydroxyacetone, previously kept in a desiccator, over phosphorus pentoxide until no further loss of weight occurs,

and making up to 100 cc. with distilled water. A few drops of toluene or xylene are added and well shaken. The solution keeps a week.

*Dihydroxyacetone 0.01 Per Cent Solution.*—This solution containing 0.1 mg. per cc. is made by diluting 1 cc. of the foregoing solution to 100 cc. with distilled water in a volumetric flask. For the preparation of the solutions containing 0.05 mg. per cc. and 0.2 mg. per cc. a 200 cc. and a 50 cc. volumetric flask is used respectively. A few drops of toluene are added. The dilute solution should be made daily.

*0.2 N  $KMnO_4$  Solution.*—This is made up in the usual way, using 6.324 gm. of pure potassium permanganate per liter of distilled water, allowing to age for a few days, filtering, and titrating against a known quantity of 0.1 N sodium oxalate solution with 5 cc. of concentrated  $H_2SO_4$  in a volume of 150 cc. at  $70^\circ C$ .

*0.01 N  $KMnO_4$  Solution.*—This solution is made fresh daily by diluting 0.2 N  $KMnO_4$  solution to twenty times its original volume. Its titer should be carefully checked by test against an 0.01 N sodium oxalate solution, freshly diluted from 0.1 N sodium oxalate solution, to which 1 cc. of 50 per cent  $H_2SO_4$  has been added, and the solution kept at  $70^\circ C$ . during the titration.

*0.1 N Sodium Oxalate Solution.*—The solution is made from United States Bureau of Standards sodium oxalate recently dried at  $110^\circ C$ . for 3 hours and kept in a desiccator. 6.70 gm. of the pure dry salt are weighed out, dissolved, and made up to a liter with water. The addition of 5 cc. of concentrated  $H_2SO_4$  facilitates solution.

*0.01 N Sodium Oxalate Solution.*—This is made by accurate ten-fold dilution of the preceding solution of 0.1 normal sodium oxalate.

*Phosphate Molybdate Solution.*—To a liter beaker containing 35 gm. molybdic acid and 5 gm. sodium tungstate add 200 cc. of 10 per cent sodium hydroxide and 200 cc. of water. Boil vigorously 20 to 40 minutes to expel ammonia, and cool. Dilute to about 350 cc. add 125 cc. of concentrated (85 per cent) phosphoric acid, and dilute to 500 cc.

#### SUMMARY.

A method for the estimation of dihydroxyacetone in pure solution, in the presence of glucose, and in the blood is described.

Examination of several hundred human blood samples fails to reveal any considerable quantity of this substance normally present in the blood. It is proposed to apply this method to the study of the metabolism of dihydroxyacetone. Dihydroxyacetone has been detected in the blood of normal individuals after the administration of the substance by mouth.

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## POTASSIUM IN ANIMAL NUTRITION.

### III. INFLUENCE OF POTASSIUM ON TOTAL EXCRETION OF SODIUM, CHLORINE, CALCIUM, AND PHOSPHORUS.\*

By HARRY G. MILLER.

*(From the Department of Agricultural Chemistry, Oregon Experiment Station, Corvallis.)*

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Factors influencing a favorable balance and ratio of inorganic constituents for physiological development are at present being extensively studied. The importance of light of definite wavelength and certain organic compounds in regulating mineral metabolism, while of recent development, has been conclusively demonstrated. The increased elimination of inorganic constituents caused by the introduction of other inorganic elements into the body fluids has for a long time received the attention of investigators. Whelan (1) has recently reviewed and contributed to this subject. The effect of potassium in this respect has claimed priority in study because of its wide occurrence in large amounts in natural foodstuffs.

The common salt requirement of man and animal subsisting on vegetable food and Bunge's early work (2) demonstrating increased urinary sodium and chlorine excretion when he added potassium salts to his diet are facts directly showing a relation between potassium intake and sodium and chlorine excretion. Bunge's work did not include results pertaining to the effect of high continued potassium intake on sodium and chlorine excretion and this phase of the problem has been reported upon by the writer (3).

Inasmuch as the former work included only a study of the urinary sodium, chlorine, and potassium the data would not permit statements on the total excretion of the above mentioned and other minerals. In the work reported here mature male rats were used

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as the experimental animals and the total excretion of calcium, phosphorus, sodium, and chlorine was determined. Certain modifications in the procedure other than the total and number of minerals excreted will not permit us to make absolute comparisons between this and the former work. For example, young growing pigs were used before, which means that there was not only a change in the type of animal but that observations were made at a different period in the life cycle. In this experiment there was a fluctuation in the weight of the animals but no greater than is found with other mature laboratory rats. The object of the present work was to study the total excretion of the minerals on a satisfactory diet and on the same diet plus added potassium salts.

#### EXPERIMENTAL.

Four mature male animals, three weighing 300 gm. each and the other 225 gm., were used. The animals were placed in separate round wire cages each with a screen bottom under which a granite pan covering the entire screen floor of the cage was placed. The porcelain cups containing the feed were held in a rack so that the tops were about 1.5 inches above the screen floor. The amount of feed scattered was negligible; however, what small quantities were not eaten were analyzed along with the excreta. All the excreta eliminated were contained in the granite pan from which they were easily washed into flasks ready for analysis.

The amount of feed which the animal would consume daily and maintain its weight was determined after which 6 or 7 days were allowed to elapse on this diet before collection of the excreta was started. The excreta were collected every 3 or 4 days. After a period of several days on the basal ration of casein 10, dextrin 84, agar 4, yeast 2, and a definite quantity of whole milk, potassium in the form of potassium acetate, potassium citrate, or potassium chloride solutions was added to the milk. The potassium solutions were made up so that 1 cc. of the solution contained about 0.1 gm. of potassium and from 1 to 2 cc. of solution were daily added to the milk during the time of high potassium intake. Milk containing a higher concentration of potassium salt was not always consumed by the animal.

Analysis of the excreta was accomplished by washing them

entirely from the pan into a 300 cc. Erlenmeyer flask with about 150 cc. of distilled water. 25 cc. of nitric acid and excess of standard silver nitrate solution were added so as to combine with all the chlorine. This solution was then digested on the steam bath until no solid particles other than silver chloride or white cellular material (agar) remained. The solution was then made to volume (250 cc.) and an aliquot, usually 50 cc., taken to determine the silver not combined with chlorine. This portion was evaporated to dryness, the organic matter burnt off, and the silver in the residue taken up with dilute nitric acid and titrated with ammonium sulfocyanate in presence of ferric alum as an indicator. For calcium, potassium, and sodium aliquots were evaporated to dryness and ashed after adding sulfuric acid. The calcium was precipitated from a solution acid with hydrochloric acid and containing ammonium chloride and ammonium oxalate. The calcium oxalate was titrated with potassium permanganate. The sulfates of sodium and potassium in the ashed residue were taken up with water, excess of barium hydroxide added, and filtered. To the filtrate excess of ammonium carbonate was added and this precipitate was filtered off. The clear liquid was evaporated to dryness and the residue was taken up with water and filtered. The filtrate was made acid with hydrochloric acid and after evaporation and just heating to dull redness the sodium and potassium were weighed as chlorides. The potassium was then determined as potassium chloroplatinate. The phosphorus was determined by ashing with magnesium nitrate, precipitating as ammonium phosphomolybdate, and finally weighing as magnesium pyrophosphate.

#### DISCUSSION.

Table I represents the average daily excretion of the different periods. Increased potassium in the diet caused an immediate rise in the total sodium and chlorine excreted similar to the increased urinary sodium and chlorine obtained with the pig. In the latter case where the rations were satisfactory for growth this increase was temporary and in comparing the periods of high and low potassium the urinary sodium and chlorine excretion was no greater during the high potassium intake. With these mature rats which were receiving a satisfactory ration but not increasing in

TABLE I.  
Average Daily Mineral Excretion.

Remarks.	Animal 115						Animal 124.					
	Received 15 cc. milk and 12.0 gm. Ration 1 daily.						Received 10.0 cc. milk and 10.00 gm. Ration 1 daily.					
	Potassium. gm.	Sodium. gm.	Chlorine. gm.	Calcium. gm.	Phosphorus. gm.		Potassium. gm.	Sodium. gm.	Chlorine. gm.	Calcium. gm.	Phosphorus. gm.	
Basal ration 6 days .....	0.0115	0.0026	0.0129	0.0153	0.0208		0.0120	0.0039	0.0097	0.0063	0.0187	
“ “ plus potassium acetate 12 days.....	0.1037	0.0077	0.0144	0.0160	0.0273		0.1054	0.0057	0.0086	0.0097	0.0208	
Increase during potassium period.....	0.0922	0.0051	0.0015	0.0007	0.0064		0.0934	0.0018	-0.0011	0.0034	0.0021	
5 days later.....												
Basal ration 6 days.....	0.0126	0.0027	0.0154	0.0193	0.0276		0.0073	0.0014	0.0123	0.0108	0.0172	
“ “ plus potassium citrate 15 days.....	0.1991	0.0079	0.0177	0.0167	0.0265		0.1942	0.0073	0.0157	0.0128	0.0212	
“ “ 9 days.....	0.0116	0.0037	0.0154	0.0207	0.0219		0.0086	0.0021	0.0120	0.0130	0.0180	
Increase during potassium citrate period.....	0.1870	0.0047	0.0023	-0.0033	0.0018		0.1863	0.0056	0.0036	0.0009	0.0036	
Basal ration plus “ chloride 6 days.....	0.1654	0.0059	0.1788	0.0218	0.0267		0.1700	0.0094	0.1808	0.0143	0.0215	
Increase during “ “ period.....	0.1533	0.0027	0.1634*	0.0018	0.0020		0.1620	0.0076	0.1687*	0.0024	0.0039	

\* 0.1808 gm. given as potassium chloride.

Ration 1: dextrin 84, casein 10, agar 4, yeast 2.

weight the total sodium and chlorine excreted was greater during the period of high potassium intake. A comparison between the two phases of experimental work is limited because the minerals in the fecal excretion of the pigs were not determined. (It may be stated however that due to the nature of the ration the intestinal elimination was very small.) Nevertheless where the pigs were on a starch diet and consequently no growth occurring, the urinary sodium and chlorine excretion was slightly greater too during the potassium salt feeding. The demands of the animal which, of course, would be greater for sodium and chlorine during growth apparently regulate the retention of sodium and chlorine during high potassium intake. In the present work the increase in the daily chlorine excretion is mostly accounted for in the first period of introducing potassium in the diet. In the following periods the total excretion approaches or is even under that occurring on the basal ration. The sodium excretion is persistently higher during the potassium feeding but a comparison of the sodium intake (0.02 gm. Animal 115 in a 3 day period) to that excreted reveals that there is a noticeable storage during the consumption of the basal ration alone which is later excreted with an increase of potassium in the diet. The depletion of the body supply of sodium and chlorine during the period of potassium feeding certainly has not gone beyond the physiological optimum. The results obtained on the total mineral excretion in this experiment substantiate our previous conclusions based upon the results obtained on urinary sodium and chlorine with the pig; namely, that the addition of potassium salts to the diet causes a temporary increase in sodium and chlorine excretion and that a continued intake of potassium does not remove these elements when they are actually needed by the animal.

The total calcium and phosphorus elimination increased during certain of the potassium periods while in others the amounts excreted were smaller than on the basal ration, leaving a small average daily increase due to excretion of excess potassium. The phosphorus increase held true when potassium chloride was given so that the increase was not apparently due to the neutralization of base resulting from the metabolism of potassium acetate or citrate. Richards and coworkers (4) report an increased assimilation of nitrogen, calcium, and phosphorus when sodium citrate,

was added to the ration of growing pigs. In mature animals such as the writer used there was not the demand for calcium and phosphorus for tissue formation and also their mineral reserves were undoubtedly built up due to the nature of the ration; therefore, a small loss of calcium and phosphorus could occur without proving injurious. The diuretic effect of potassium chloride as reported by Whelan (1) may have caused the increased elimination of these elements. Potassium does not always cause an immediate increase in calcium and phosphorus excretion as has been observed with sodium and chlorine.

The amount of potassium in proportion to body weight was extremely high in the ration of these rats. The quantity of potassium in the feed of a herbivorous animal like the cow will not, according to our observations, go over 1 part of potassium to 2200 parts of body weight. In our experimental pigs the ratio was about 1 to 3000, while with the rats it varied from 1 part of potassium to 1200 or 1500 parts of body weight. By supplementing the ration with these potassium salts one would suppose that conditions are more favorable for larger quantities of potassium entering the circulation than when the potassium is supplied in the form in which it occurs in natural foodstuffs. Opinions similar to this have appeared in the literature. However, we have observed that 90 to 95 per cent of the potassium in the ration of a dairy cow is absorbed from the alimentary tract. Plans to obtain data similar to these for the rat are being contemplated. Considering the quantity of available potassium these animals were subjected to the influence of as high or higher concentration of potassium salts than is ordinarily found in the feed of animals or food for human consumption.

#### SUMMARY.

1. The effect of high potassium on the total excretion of sodium, chlorine, calcium, and phosphorus with mature rats receiving a ration of casein, dextrin, agar, yeast, and milk has been studied over periods of 2 weeks duration.

2. The introduction of potassium salts in the diet caused an immediate increase in total sodium and chlorine excreted after which the amount of these elements excreted was only slightly greater than on the basal ration.

3. During certain periods of high potassium feeding the total calcium and phosphorus excreted increased a small amount compared to a like period on the basal ration; however, the average daily excretion of calcium and phosphorus was slightly increased over the low potassium period.

4. Considering the abnormally high level at which potassium was fed in this experiment and results obtained in previous experiments there is no reason to believe that potassium occurring in natural foodstuffs will cause an increased excretion of other elements when they are needed for normal physiological development.

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## THE OCCURRENCE OF UREASE IN THE BLOOD CELLS, BLOOD PLASMA, AND TISSUES OF LIMULUS.

BY LEO LOEB AND OSCAR BODANSKY.

(From the Department of Pathology, Washington University, St. Louis,  
and the Marine Biological Laboratory, Woods Hole.)

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While studying the effect of non-electrolytes on the behavior of amebocyte tissue in tissue cultures, we noticed after addition of urea to the fluid surrounding the tissue a considerable shifting of the pH towards the alkaline side, the end-point reached being approximately pH 9.0 or 9.1. This suggested to us the possibility that urease might be present in the amebocytes and we carried out a series of experiments in which we tested this suggestion.

We found that urease was present not only in the amebocytes of *Limulus*, but also in the blood plasma (blood serum), in the muscle tissue and in the eggs of *Limulus*, therefore in all the tissues and body fluids of *Limulus* which we have tested so far, while in the corresponding tissues and fluids of somewhat related animals like lobster and spider-crab the presence of urease could not be demonstrated. For the determination of the urease action we used in our experiments the methods of Folin,<sup>1</sup> Marshall,<sup>2</sup> and Van Slyke.<sup>3</sup> It may be stated that amebocyte tissue is prepared by allowing amebocytes *in vitro* to agglutinate under sterile conditions which avoid as much as possible injurious changes in the cells and which lead to the formation of a tissue-like layer at the bottom of the dish in which the blood of *Limulus* has been received.<sup>4</sup> Inasmuch as a true coagulation of fibrinogen does not occur in

<sup>1</sup> Folin, O., *Z. physiol. Chem.*, 1902, xxxvii, 161; A laboratory manual of biological chemistry, New York and London, 3rd edition, 1923.

<sup>2</sup> Marshall, E. K., *J. Biol. Chem.*, 1913, xv, 487; 1914, xvii, 351.

<sup>3</sup> Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141; 1916, xxiv, 117.

<sup>4</sup> Loeb, L., *Washington Univ. Studies*, 1920, viii, 5; *Am. J. Physiol.*, 1921, lvi, 140. Loeb, L. and Blanchard, K. C., *Am. J. Physiol.*, 1922, lx, 277.



*Limulus* blood,<sup>6</sup> the usual distinction between blood plasma and blood serum does not hold good in this case, but we shall designate as blood serum the fluid remaining in the blood after agglutination of the amebocytes has taken place.

*Presence of Urease in Amebocyte Tissue and the Temperature at Which Urease Is Destroyed in Amebocyte Tissue.*—Preliminary experiments showed that amebocyte tissue kept under apparently sterile conditions in contact with solutions of urea for 24 hours in a stoppered flask developed a strong odor of ammonia; in contact with water instead of urea solution this odor did not develop. Bacteriological examinations (smear and culture) failed to show the presence of bacteria. In all subsequent experiments toluene was added to the mixture of the amebocyte tissue or other material tested for urease. The urea (Kahlbaum or Baker) was made up in the strength of 0.97 M which is approximately isotonic with sea water. The amount of amebocyte tissue added varied in weight between about 0.5 and 1.0 gm.; it was in every case as carefully as possible freed from adhering serum.

In control experiments carried out with Van Slyke's method, in which only solutions of urea and sodium carbonate were present in the aerating tube, it was found that about 19.00 to 19.75 cc. of 0.01 N NaOH would neutralize 20.00 cc. of 0.01 N HCl. This error of about 0.50 cc. may be due to alkalinity of the glass tubes or the spurting over of  $\text{Na}_2\text{CO}_3$  from the aerating tube. The apparatus used in these experiments had to be prepared by us.

A mixture of amebocyte tissue, 10 cc. of  $\text{H}_2\text{O}$ , 10 cc. of urea solution, and a few drops of toluene in a flask was allowed to remain at room temperature for 24 hours; 5 cc. of the mixture were used for the test. After treatment by Folin's method,<sup>1</sup> 10 cc. of 0.1 N HCl required 2 cc. of 0.1 N NaOH for neutralization. Ammonia neutralized therefore 8 cc. of HCl.

If in a similar mixture instead of fresh amebocyte tissue, amebocyte tissue that had previously been boiled for 5 minutes was used, 10 cc. of 0.1 N HCl required 9.65 cc. of 0.1 N NaOH for neutralization. Therefore boiling had destroyed the urease.

If the tissue had been kept in water at 70° for 15 minutes before being added to urea, the urease was still active. The tissue was

<sup>6</sup> Loeb, L., *Biochem. Z.*, 1910, xxiv, 478.

added to 10 cc. of water, 15 cc. of urea solution, and a few drops of toluene. After standing for 46 hours, the filtrate of this mixture was used for determination of urease. 20 cc. of 0.1 N HCl required 0.90 cc. of 0.1 N NaOH for neutralization; hence 19.10 cc. were neutralized by ammonia. If in a corresponding experiment tissue heated to 80° was used, 10 cc. of 0.1 N HCl required 9.20 cc. of 0.1 N NaOH for neutralization. Therefore heating of the tissue to 80° 15 minutes had destroyed the urease.

Similar results were obtained with Marshall's method.

*Tissue Previously Heated to 60° for 30 Minutes.*—To 10 cc. of filtrate 30 cc. of 0.1 N HCl were added; 6.75 cc. of 0.1 N NaOH were required for neutralization; hence 23.25 cc. of acid had been neutralized by ammonium carbonate.

*Tissue Heated to 70° for 30 Minutes.*—To 10 cc. of filtrate 20 cc. of 0.1 N HCl were added. 7.70 cc. of 0.1 N NaOH were required for neutralization. Hence 12.30 cc. of acid had been neutralized by ammonium carbonate.

*Tissue Heated to 80° for 30 Minutes.*—To 10 cc. of filtrate 10 cc. of N HCl were added. 10 cc. of 0.1 N NaOH were required for neutralization. No ammonium carbonate was present. Urease had been destroyed through heating to 80°.

We may conclude from these experiments that urease of amebocyte tissue of *Limulus* is destroyed at a temperature ranging somewhere between 70° and 80°. This agrees with the results of Van Slyke, who obtained inactivation at a similar temperature in the case of the soy bean urease.<sup>3</sup>

*Reaction in the Solutions Which Contain Urease.*—In our tissue culture experiments, in which urea had been added to the fluid surrounding the amebocyte tissue, the reaction was tested usually after about 24 hours and an end pH of approximately 9.0 was always obtained, independently of whether or not the urease acted in a urea solution to which acid or alkali had previously been added. In the following experiments we used the same proportions of substances as in the tissue culture experiments.

*Experiment A.*—To pieces of amebocyte tissue in flask were added 15.88 cc. of H<sub>2</sub>O, 5.62 cc. of 2 N NaCl, 1 cc. of 0.1 N NaOH, 7.5 cc. of 0.97 M urea, and 3 drops of toluene. This is a solution of 1 part urea and 3 parts N/2 NaCl in N/300 NaOH with a pH of 9.2. At the end of 24 hours the pH of the solution was 8.9. To

10 cc. of the filtered fluid 10 cc. of 0.1 N HCl are added and the mixture is titrated with 0.1 N NaOH. With methyl orange as indicator 3.80 cc. of alkali were required for neutralization. Therefore 6.20 cc. of the acid had been neutralized by ammonium carbonate. When heated tissue was used instead of fresh tissue, no ammonium carbonate was formed and the original pH 9.2 remained unchanged.

In tissue culture experiments the urease action took place apparently even in solutions to which enough HCl had been added to cause an acid reaction in the mixture; this was confirmed in titration experiments (Marshall's method). A solution of 1 part of 0.97 M urea, 3 parts of N/2 NaCl, and 3 drops of toluene in N/200 HCl was prepared. pH = 4.0. After 24 hours standing, the pH had changed to 9.1. 15 cc. of 0.1 N HCl were added to 10 cc. of the filtered mixture. It was found that 11.55 cc. of the acid had been neutralized by ammonium carbonate. In a control experiment in which the tissue had first been heated, no ammonium carbonate was produced.

A similar end pH had been observed by Van Slyke<sup>3</sup> in his experiments with the soy bean urease, and this pH was attributed by this author to the presence of ammonium carbonate, the end-product of urease action.

In all the following experiments Van Slyke's method was used with one exception which will be mentioned.

*Urease in the Blood Serum of Limulus.*—The blood serum of *Limulus* contains quantities of urease which vary in different animals. We may cite the extremes which we found. (a) 7.5 cc. of serum, 10 cc. of urea, and toluene stood at room temperature for 24 hours. From 10 cc. of this mixture ammonia was obtained sufficient to neutralize 4.80 cc. of 0.01 N HCl. (b) From 10 cc. of a similar mixture, but in which a different kind of serum had been used, an amount of ammonia was obtained sufficient to neutralize 56.80 cc. of 0.01 N HCl. Through heating serum to 80° for 30 minutes the urease is to a great extent (but perhaps not yet entirely) destroyed. From 10 cc. of a similar mixture, as used above, in which the serum was unheated, enough ammonia was obtained to neutralize 4.80 cc. of 0.01 N HCl. After heating the serum, only 1.40 cc. of 0.01 N HCl was neutralized.

We made approximate tests as to the relation between the

amounts of serum used and the quantity of ammonia which developed in the mixture of 10 cc. of urea solution and varying amounts of serum, the whole being made up to 25 cc. by the addition of varying amounts of water.

	Serum (acting on urea for 24 hrs.).	In 10 cc. of the mixture, amount of 0.01 N HCl neutralized by ammonia after addition of 25 cc. 0.01 N HCl.
	cc.	cc.
Experiment A.	0	0.65
	1	2.15
	3	5.45
	5	2.10
	10	9.45
	15	18.30
Experiment B.	0	0.20
	0.5	0.90
	1	2.10
	3	2.60
	5	5.55
	10	13.20

We notice that small amounts of serum up to 1 or 3 cc. produce very small amounts of ammonia, and that the increase in ammonia is relatively slight between 1 and 5 cc. of serum, but that it increases considerably between 5 and 10 or 15 cc. In later experiments we hope to determine more accurately the curve of ammonia production in dependence on the amount of serum added.

*Urease in Muscle of Limulus.*—After having found the presence of urease in amebocytes and in the blood serum of *Limulus*, it was of interest to determine whether other tissues of *Limulus* likewise contained urease. We tested, therefore, the muscle and the eggs of *Limulus* for urease action in the same way as we did amebocyte tissue.

A mixture of 3 gm. of *Limulus* muscle, 10 cc. of H<sub>2</sub>O, 10 cc. of urea solution, and toluene was allowed to remain at room temperature for 24 hours. In 5 cc. of the filtered fluid ammonia neutralized 10.75 cc. of 0.01 N HCl. In a second experiment in which a similar mixture remained at room temperature for 52 hours, the liberated ammonia neutralized 52.85 cc. of 0.01 N HCl.

In a control in which 3 gm. of *Limulus* muscles were allowed to

remain for 52 hours in a mixture of 20 cc. of  $H_2O$  and toluene without urea only 2.05 cc. of 0.01 N HCl were neutralized by ammonia and in a second experiment with different *Limulus* muscle after 25 hours 1.80 cc. of 0.01 N HCl were neutralized. In both cases the very slight amount of neutralization which had taken place was due to other factors than urease action. As we shall see later lobster muscle does not contain urease.

*Urease in Eggs of Limulus.*—A mixture of 10 cc. of water, 10 cc. of urea solution, toluene, and 3 gm. of unfertilized eggs of *Limulus* was allowed to remain for 24 hours at room temperature. In 5 cc. of filtered fluid ammonia had neutralized 16.55 cc. of 0.01 N HCl. In another experiment in which the eggs of a different *Limulus* were used, ammonia had neutralized 54.65 cc. of 0.01 N HCl after the mixture had been standing at room temperature for 45 hours. In each case the eggs were carefully dried with filter paper and all adhering material was removed.

In a control experiment with boiled eggs of *Limulus* 3.25 cc. of 0.01 N HCl were neutralized after the mixture had been allowed to remain for 45 hours, and in a mixture with fresh eggs, 20 cc. of  $H_2O$  and toluene, but no urea solution, 1.65 cc. of 0.01 N HCl were neutralized. In both cases urease action was lacking.

We may then conclude that in muscle as well as in unfertilized eggs of *Limulus* urease is present. The urease evidently does not develop in the course of embryonal development, its formation does not depend on the formation of certain organs, but it is apparently preformed in the ova. There is still the possibility that the urease is not produced within the eggs but is carried to the eggs by the blood and is merely stored by the eggs. While this is not probable, it will be necessary to test this possibility experimentally.

*Is Urease Present in the Blood or Muscle of Crustaceans?*—We tested in a similar way to *Limulus* amebocyte tissue and *Limulus* blood serum, the blood serum and amebocytes of the lobster and spider-crab; the blood of several animals was used for each experiment. The blood was shaken while it was received in a dish. Thus the agglutinated amebocytes were separated at once from the blood plasma. The blood of these animals contains fibrinogen,<sup>6</sup> and therefore it is possible that a very slight amount of

<sup>6</sup>Loeb, L., *Beitr. chem. Physiol. u. Path.*, 1904, v, 191; 1907, ix, 185; 1905, vi, 260; 1906, viii, 67.

fibrin might have been admixed to the agglutinated amebocytes in this case. The amebocytes as well as the plasma of lobster and spider-crab were found to lack urease entirely, less than 1 cc. of 0.01 N HCl being neutralized by the filtered fluid obtained from mixtures similar to the ones used in the case of *Limulus* tissue or blood serum. Similarly the muscle of lobster behaved like *Limulus* muscle in the control experiments in which no active urease was present; in a mixture of 3 gm. of lobster muscle, 10 cc. of H<sub>2</sub>O, 10 cc. of urea solution, and toluene only 1.60 cc. of 0.01 N HCl had been neutralized. Urease action was therefore absent in blood and tissues of crustaceans tested so far.

These experiments thus indicate that *Limulus* is sharply differentiated from marine crustaceans through the character of the enzymes which are active in its tissue and organs. It is assumed that *Limulus* is phylogenetically more nearly related to the arachnoids than to the crustaceans; it would therefore be of interest to search among other classes of arthropods and especially among those more nearly related to *Limulus* for the presence of urease in the blood cells and in the various tissues. Przylecki<sup>7</sup> found a relatively weak urease action in the liver of *Astacus* while its muscle was found unaffected. In mollusks the occurrence of urease seems to be more common according to the findings of Przylecki and of Albrecht.<sup>8</sup>

*Extraction of Urease from the Amebocyte Tissue.*—The fact that urease is found both in amebocytes and blood serum of *Limulus* suggests that the enzyme in the serum (plasma) is derived from the amebocytes. We made, therefore, experiments in which we attempted to determine the conditions under which urease can be extracted from amebocyte tissue.

(a). In the first experiment we received about 35 cc. of blood of *Limulus* in each of several flasks in a sterile manner. A layer of amebocyte tissue formed at the bottom of each flask. Thus there was a chance for the supernatant serum to extract additional urease from the amebocyte tissue. The flasks were kept in the ice chest and at various periods the serum was poured off and tested in the usual way for the strength of urease action. In addition a sample of blood was immediately, after it was obtained from the animal,

<sup>7</sup> Przylecki, H. J., *Arch. internat. physiol.*, 1922, xx, 103.

<sup>8</sup> Albrecht, P. G., *J. Biol. Chem.*, 1920-21, xlv, 395.

freed from the amebocytes through centrifuging and filtering, and likewise tested for urease action. All the samples of blood serum, those tested immediately, as well as after standing 52 and 144 hours in contact with amebocyte tissue, showed approximately the same strength. For testing the urease content a mixture of 7.5 cc. of serum, 10 cc. of urea solution, and a few drops of toluene was prepared. In 10 cc. of the mixture ammonia was produced sufficient to neutralize amounts of 0.01 N HCl which varied in the different samples between 9.40 and 12.65 cc.

In another experiment in which a similar series of tests was made with the blood of a different *Limulus* the urease content was much stronger but again the different samples behaved similarly: 10 cc. of the mixture neutralized between 50 and 60 cc. of 0.01 N HCl irrespective of the time during which the serum had been in contact with the amebocyte tissue, which extended up to 6 days. We may conclude from these experiments that the urease is present in the blood plasma of *Limulus* within the living animal and is not merely extracted *in vitro*. Our method of collecting the blood tended to preserve the amebocytes intact. These experiments do not however prove conclusively that no urease can be extracted from the amebocytes by normal *Limulus* serum. It is possible that after a relative state of saturation with urease has been reached in the serum, further extraction is difficult. It will be necessary to test these questions in further experiments.

(b). While the preceding experiments did not prove an extraction of urease from amebocyte tissue by serum, it was possible to prove definitely an extraction of urease by *Limulus* serum after the urease of that serum had previously been destroyed by heating the serum up to 80°C. for 30 minutes. While 10 cc. of a mixture of 7.5 cc. of unheated serum with 10 cc. of urea solution and several drops of toluene after standing for 24 hours produced sufficient ammonia to neutralize 4.80 cc. of 0.01 N HCl, a similar mixture with heated instead of unheated serum, neutralized only 1.40 cc. of 0.01 N HCl. To each of two samples of the heated serum was then added a piece of fresh amebocyte tissue, 1 to 2 gm. of tissue being added to one sample and less than 1 gm. to the second sample. After standing 1 day with the addition of toluene, 12 cc. of each sample were mixed with 10 cc. of urea solution and toluene

and allowed to act for 24 hours. After this time it was found that in the first mixture to which a larger amount of amebocyte tissue had been added, 10 cc. of the mixture, containing 5.5 cc. of serum, ammonia was produced which neutralized 27.25 cc. of 0.01 N HCl, and in the second mixture in which a smaller amount of amebocyte tissue had been added the same amount of serum had produced ammonia in sufficient quantity to neutralize 7.05 cc. of 0.01 N HCl. We may then conclude that the urease content of the heated serum increased considerably after contact with the amebocyte tissue and that urease had been extracted from the tissues. This experiment was repeated with similar results.

(c). *Extraction of Urease from Amebocyte Tissue by Other Fluids.*—In this experiment 20 cc. of each of several fluids to which were added a few drops of toluene were kept in small flasks in contact with weighed quantities of amebocyte tissue for 48 hours. The fluids were then poured off and filtered and 10 cc. of urea solution were added to each; these mixtures were allowed to remain for 24 hours and tested for ammonia; 10 cc. samples of the filtrates were used. Experiments I and II show the amount of ammonia which had been produced in the mixture. From this figure we must deduct approximately 0.5 cc. which may be due to other sources than ammonia produced by urease extracted from the amebocyte tissue.

## EXPERIMENT I.

Character of fluid used for extraction.	Weight of amebocyte tissue.	Amount of 0.01 N HCl neutralized by ammonia after aeration of the mixture of filtered fluid and urea solution. <sup>g</sup>
	mg.	cc.
Distilled water.....	510	0.50
N/2 NaCl solution .....	1700	1.10
Sea water (pH 9.0).....	500	2.10
“ “ (previously neutralized).	425	2.10
Serum from which the protein had been removed through heating and filtration (serum filtrate)....	570	4.40



## EXPERIMENT II.

Time of extraction of amebocyte tissue 24 hrs.

Character of fluid used for extraction.	Weight of amebocyte tissue.	Amount of 0.01 N HCl neutralized by ammonia after aeration of the mixture of filtered fluid and urea solution.
	mg.	cc.
Distilled water .....	500	0.85
Sea water (alkaline) .....	500	0.50
N/2 NaCl solution .....	500	0.40
Serum filtrate .....	500	0.80
Heated serum .....	500	4.80

From these experiments we may conclude that fluids other than heated blood serum of *Limulus* tested so far either extract none or only very small amounts of urease from amebocytes or that if the urease should be extracted it is somehow inactivated soon after extraction. It still remains to be determined whether different fluids differ somewhat in their extracting or preserving capacity, with the possibility that those fluids are more effective which approach in composition the blood serum of *Limulus*. The result of these experiments was confirmed by two other experiments in which we attempted to disintegrate the amebocyte tissue before extraction. At first we tried to grind the tissue with sand. Subsequent addition of water to this material failed to extract the enzyme; the enzyme remains however in the solid material and can here be demonstrated by its action on urea solution.

In another experiment a powder was prepared of amebocyte tissue. For this purpose the tissue was dried with filter paper, placed in 95 per cent alcohol for 30 minutes; the alcohol was poured off and the remaining material was treated with ether. After the fatty substances had been extracted and the ether removed, the remnants of amebocyte tissue could be ground up into a fine white powder which showed urease action, about 100 mg. of the powder in contact with 10 cc. of urea solution producing in 24 hours sufficient ammonia to neutralize more than 30 cc. of 0.01 N HCl. However when this powder was extracted with distilled water for several hours, the watery extract proved to be inactive, while the extracted powder continued to show urease action. Again we find that water is apparently either unable to extract urease from the

amebocyte tissue or it destroys the extracted enzyme within a short time.

As to the physiological function of the urease in *Limulus* no statement can be made at the present time. In the blood of the crustacean *Panulirus*, Morgulis discovered a small amount of urea;<sup>9</sup> but in *Limulus* Morgulis<sup>10</sup> determined so far only the amount of non-protein nitrogen which varied between 26 and 10 mg. per 100 cc. of blood plasma; it was greatest in freshly caught animals and decreased gradually in individuals kept in tanks under conditions of deficient nourishment.

There are, however, some unpublished experiments of Morgulis<sup>11</sup> which we could interpret as indicating activity of the urease also in the living animal: An injection of 1 gm. of urea in sea water into the circulation of *Limulus* invariably killed *Limulus* of medium size. Even 0.5 gm. of urea caused the death of the animal, but usually this occurred after a lapse of many hours, sometimes 12 to 18 hours. But with the 1 gm. dose limuli may die within an hour and with larger doses still in a much shorter time. The injected limuli show tetanic contraction of the muscles. Injection of similar quantities of urea in spider-crabs was without effect.

We may conclude that the urease present in the blood and tissues of limuli caused the production of ammonia in the blood and tissue and that this substance was responsible for the muscular contractions and the death of the animal. These observations are in accordance with the previous experiments of Carnot and Gerard,<sup>12</sup> who found similar effects after urease injections in dogs. The injection of urease diminished the urea level in the blood and caused the production of ammonia. Lövgren<sup>13</sup> also recorded toxic effects after injection of urease. On the other hand, Lublin<sup>14</sup> observed that subcutaneous injection of urease in mice and rabbits was without effects on the well being of the animals. Intravenous injection caused death, which this author however attributes to

<sup>9</sup> Morgulis, S., *Ann. Rep., Dept. Marine Biol., Carnegie Institution Year Bk., No. 21*, 173.

<sup>10</sup> Morgulis, S., *J. Biol. Chem.*, 1922, 1, p. lii.

<sup>11</sup> Personal communication of Dr. Morgulis.

<sup>12</sup> Carnot, P., and Gerard, P., *Compt. rend. Acad.*, 1919, clxix, 88.

<sup>13</sup> Lövgren, S., *Biochem., Z.*, 1921, cxix, 215.

<sup>14</sup> Lublin, A., *Arch. exp. Path. u. Pharmacol.*, 1922, xcii, 280.

thrombi produced through agglutination of erythrocytes. But after simultaneous subcutaneous injection of urease and urea solution death occurred with symptoms of ammonia poisoning also in these animals.

#### SUMMARY.

1. In the amebocytes, in muscle tissue, in the unfertilized eggs, and in the blood plasma of *Limulus* urease is present in considerable amounts. We must assume that the urease is either preformed in the eggs of *Limulus* and does not depend on the development of certain organs or tissues for its formation or else that ova have the power of absorbing the enzyme from the blood plasma. The greater part of the enzyme is destroyed by heating the tissue or serum to a temperature ranging between 70° and 80°C. for about 30 minutes.

2. Urease can be extracted from amebocyte tissue by blood serum of *Limulus*, after the serum has been deprived of its urease by previous heating. Other fluids tested so far extract the urease from the amebocyte tissue either not at all or only very slightly or perhaps these fluids inactivate the extracted enzyme very rapidly. There is some indication that possibly those fluids are most effective in the extraction of the urease which approach the blood serum of *Limulus* in their constitution.

3. From our experiments we may conclude that the urease of the blood plasma is to a large extent derived from the amebocytes. We did not find urease in the blood or tissues of other crustaceans which we tested so far. *Limulus* seems thus to be differentiated from certain other classes of arthropods by its enzymotic constitution.

4. The toxic effects of the injection of urea in *Limulus* observed by Morgulis are probably due to the action of the urease in the living animal.

## THE EFFECT OF HISTAMINE ON THE BLOOD CHLORIDES.\*

BY T. G. H. DRAKE AND FREDERICK F. TISDALL.

*(From the Nutritional Research Laboratories of the Hospital for Sick Children and the Sub-Department of Pædiatrics, University of Toronto, Toronto, Canada.)*

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Much work has been done in recent years on the intoxication of intestinal obstruction. It is not our intention to enter into any discussion of the probable cause of the intoxication beyond drawing the attention of the reader to the fact that some investigators have suggested that histamine may be the toxic element (1, 2). The evidence in support of this belief is that histamine has been isolated from the intestinal mucosa, the intestinal contents, and from the contents of isolated closed intestinal loops (1, 2, 3). Also the symptoms produced by the injection of histamine in animals are similar in many respects to those produced by the toxin obtained from isolated intestinal loops (4).

A marked decrease in the chlorides of the blood after the experimental production of high intestinal obstruction or obstruction of the pylorus has been noted by MacCallum, Lintz, Vermilye, Leggett, and Boas (5), Hastings, Murray, and Murray (6), Ingvaldsen, Whipple, Bauman, and Smith (7), and Haden and Orr (8). A similar drop in blood chlorides after intestinal obstruction in humans has been observed by Brown, Eusterman, Hartman, and Rowntree (9), Orr and Haden (10), Gamble, Ross, and Tisdall (11), Tisdall (12), and others.

In view of these findings it is of considerable interest to determine whether the injection of pure histamine produces any reduction in the concentration of the blood chlorides. The only values

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TABLE I.  
*Effect of Repeated Subcutaneous Injections of Histamine (Ergamine Acid Phosphate) on the Concentration of Plasma Chlorides of Dogs.*

Dog No.	Weight of dog.	Histamine administered per lb. of body weight.	Total histamine administered.	Amount and time of administration of histamine.	Interval between removal of samples of blood.	Plasma chlorides before.	Plasma chlorides after.	Remarks.
	lbs.	mg.	mg.	mg. per lb.	hrs.	mg. Cl per 100 cc.	mg. Cl per 100 cc.	
17	12	17.5	210	2.5, 3 p.m., 11 p.m., 9 a.m., 10 at 11 a.m.	24		290	Slightly toxic.
20	18	20.0	360	5.0, 10 a.m., 5 p.m., 11 p.m., 10 a.m.	28	360	315	" "
21	14	17.5	245	7.5, 9 a.m., 5.0, 5 p.m., 10 p.m.	12	380	315	Very toxic. Died during night.
22	15	7.5	112	7.5, 9 a.m.				Died in 6 hrs.
24	16	9.5	152	3.5, 9 a.m. 2.0, 5 p.m. 1.0, 9 " 2.0, 9 a.m. 1.0, 12 "	29	385	325	Apparently normal.
26	17	14.0	238	4.0, 9 a.m., 1 p.m. 4 p.m. 2.0, 7 p.m.	12	380	355	Very toxic.

27	20	16.0	320	4.0, 9 a.m., 12 noon, 3 p.m., 6 p.m.	12	380	345	Very toxic.
31	15	12.0	180	4.0, 9 a.m., 1 p.m., 5 p.m.				Died in 10 hrs
32	16	16.0	256	4.0, 9 a.m., 1 p.m., 5 p.m., 9 p.m.	12	405	380	Very toxic. Died dur- ing night.
33	17	6.0	102	4.0, noon. 2.0, 11 p.m.	12	370	320	" " "
34	20	4.0	80	4.0, noon.				Died during evening.
35	13	10.0	130	2.0, noon. 4.0, 11 p.m., 9 a.m.	24	380	330	Apparently normal.
36	16	10.0	160	2.0, noon. 4.0, 11 p.m., 9 a.m.	24	380	340	" "
37	11	10.0	110	2.0, noon. 4.0, 11 p.m., 9 a.m.	24	365	345	" "
38	11	10.0	110	2.0, noon. 4.0, 11 p.m., 9 a.m.	24	365	355	" "
Average values.....						377	334	

*the authors have been able to find in the literature are those recently recorded by Hashimoto (13).* This investigator, working with dogs, gave intravenous injections of 1 to 3 mg. of histamine dichloride dissolved in small amounts of physiological salt solution and maintained the intoxication for periods of from 3 to 5 hours by similar injections every 30 to 60 minutes. The blood chlorides were determined at intervals up to 24 hours after the injections. No consistent variation in the concentration of the blood chlorides was observed.

In the present study the authors administered histamine (Pfansteil) (ergamine acid phosphate) subcutaneously to dogs. The amounts given were very much larger than those used by Hashimoto, and they were also administered over a longer period. Consequently the intoxication was more prolonged than that obtained by him. Most of the animals used were pups although their exact age was not known. It was soon evident that the susceptibility of different dogs to the effects of histamine varied tremendously, but apparently without relation to the age of the animals. A study of Table I shows that some animals did not survive a single dose of 4 mg. per pound, while others were not very toxic even when large doses were given repeatedly.

The blood chlorides were determined on the plasma by the method of Whitehorn (14). The determinations were made on the plasma rather than the whole blood since the concentration of the chlorides in the corpuscles is very much less than the plasma. Consequently any increase in the percentage of corpuscles would result in a decrease in the percentage of chlorides when determined on the whole blood, without any change in the percentage of chlorides in the plasma. The dogs were not fed for some hours before, or during the course of the experiment. Water was offered at intervals. Table I shows a consistent drop in the concentration of plasma chlorides after the administration of histamine.

In Table II is given the concentration of the plasma chlorides at various intervals during the course of the experiment. These results lend support to Hashimoto's conclusion that a histamine intoxication lasting from 3 to 5 hours produces no consistent change in the plasma chlorides. Nevertheless when the intoxication is prolonged the concentration of plasma chlorides is progres-

sively diminished. There is no evidence however that the degree of intoxication bears any relation to the concentration of the plasma chlorides.

TABLE II.

*Effect of Repeated Subcutaneous Injection of Histamine (Ergamine Acid Phosphate) on the Concentration of Plasma Chlorides and Plasma Non-Protein Nitrogen at Intervals during the Course of the Experiment.*

Dog No.	Weight of dog.	Amount and time of administration of histamine.	Time of removal of blood and concentration of plasma chlorides.	Concentration of plasma non-protein nitrogen.
	lbs.	mg. per lb.	mg. per 100 cc.	mg. per 100 cc.
16	27	7.0, 9 a.m.	9 a.m., 385 10 " 395 11 " 390 12 noon, 395 1 p.m., 385 2 " 390 3 " 375	.
20	18	5.0, 10 a.m. 5.0, 5 p.m. 5.0, 11 " 5.0, 10 a.m.	10 a.m., 360 10 p.m., 325 2 " 315	10 a.m., 18.0 10 p.m., 20.0 2 " 26.0
21	14	7.5, 9 a.m. 5.0, 5 p.m. 5.0, 10 "	9 a.m., 380 3 p.m., 380 6 " 335 10 " 315	9 a.m., 17.1 3 p.m., 24.0 6 " 27.2 10 " 85.7
24	16	3.5, 9 a.m. 2.0, 5 p.m. 1.0, 9 " 2.0, 9 a.m. 1.0, 12 noon.	9 a.m., 385 3 p.m., 355 6 " 325 2 " 325	9 a.m., 23.0 3 p.m., 26.0 6 " 22.2 2 " 18.1
27	20	4.0, 9 a.m. 4.0, 12 noon. 4.0, 3 p.m. 4.0, 6 "	9 a.m., 380 5 p.m., 360 9 " 345	9 a.m., 20.0 5 p.m., 52.0 9 " 60.0

The degree of toxemia appears to be closely related to the concentration of the non-protein nitrogen. Dog 24 which was apparently normal showed practically no increase in the non-protein nitrogen. Dog 20 which was only slightly toxic showed only a



slight increase. On the other hand Dogs 21 and 27 which were very toxic showed a marked increase in the non-protein nitrogen.

On account of the vomiting resulting from obstruction of the intestine it has been suggested by MacCallum and his coworkers (5), Hastings, Murray, and Murray (6), Ingvaldsen and coworkers (7), and others that the low concentration of blood chlorides found in intestinal obstruction is caused by a loss of chlorine in the vomitus. Haden and Orr (8) advance the interesting theory that chlorine leaves the blood stream for the tissues as a protective agent against the toxic substance. They consider that a loss of chlorine in the vomitus is not a factor since a low concentration of blood chlorides occurred in monkeys and rabbits (8) after intestinal obstruction. These animals did not vomit. Although no vomiting occurred with rabbits Gamble and McIver (15) have demonstrated that large quantities of gastric secretion may collect in the stomach. This secretion from the metabolic standpoint is outside the body as effectually as though it had been vomited.

Histamine has been shown to stimulate the secretion of gastric juice, particularly the hydrochloric acid (16, 17, 18, 19). In view of this fact and the suggestion of many observers that the low blood chlorides of intestinal obstruction are the result of the vomiting, it is of considerable interest to determine the importance of loss of gastric secretion as a factor in the reduction of the blood chlorides after the administration of histamine. Accordingly the following experiments were performed. Dogs were placed in cages with a harness arranged so that the vomitus would be caught in a receptacle. A sample of blood about 10 cc. was removed from the animal's heart and histamine then given subcutaneously at different intervals. The vomitus was collected and at the conclusion of the experiment the stomach was washed out, after the final sample of blood had been procured. It was soon found that washing out the stomach was a difficult procedure so it was replaced later by the subcutaneous administration of 1 mg. of apomorphine per kilo of body weight. This produced marked vomiting in about 5 minutes. The total vomitus was measured and filtered. Proteins were removed by boiling with a few drops of acetic acid. The amount of protein was so extremely small however that the procedure was later omitted. The chlorides

were estimated directly on the filtrate by the method of Whitehorn (14). The percentage of corpuscles was determined in the blood samples by centrifuging the blood at high speed for over 1 hour in graduated centrifuge tubes. The percentage of protein in the

TABLE III.

*Effect of Repeated Subcutaneous Injection of Histamine (Ergamine Acid Phosphate) on the Amount of Chlorine Lost in the Gastric Secretion, Also Effect on Percentage of Corpuscles in the Blood and Percentage of Protein in the Blood Serum.*

For the details of the amount of histamine given and the duration of each experiment see Table I.

Dog No.	Plasma chlorides before.	Plasma chlorides after.	Total chlorine in vomitus.	Chlorine in vomitus per lb. of body weight.	Corpuscles in blood before.	Corpuscles in blood after.	Serum protein before.	Serum protein after.
	mg. Cl per 100 cc.	mg. Cl per 100 cc.	mg.	mg.	per cent	per cent	per cent	per cent
20	360	315			52.0	47.0	6.18	5.98
21	380	315			56.0	46.5	5.47	6.34
24	385	325			47.7	47.4	5.90	6.77
26	380	355	268	15.7	45.2	55.7	4.94	4.38
27	380	345	1300	65.0	69.3	65.1	5.90	5.68
32	405	380	279	17.5	59.0	48.5	5.58	5.47
33	370	320	922	54.2	41.0	40.0		
35	380	330	420	32.3	56.8	41.5	4.38	5.53
36	380	340	688	43.0	48.7	42.5	6.25	6.55
37	365	345	58	5.2	47.5	54.0	6.77	7.20
38	365	355	74	6.7	47.0	45.2	6.98	7.20
Average, Dogs 26 to 38.....	378	346		29.9				
Average for all dogs.....					51.8	48.4	5.83	6.11

blood serum was also determined at the commencement and conclusion of each experiment. The results are given in Table III.

Control experiments were carried out with apomorphine. 1 mg. of apomorphine per kilo of body weight was subcutaneously administered at intervals. With Dogs 25 to 30 apomorphine was

administered at 9 a.m., 12 noon, 3 p.m., and 6 p.m. Samples of blood were removed at 9 a.m. and 9 p.m. After the blood was removed at 9 p.m. another injection of apomorphine was given to empty the stomach. With Dogs 39 to 44 the injections were given at noon, 5 p.m., 11 p.m., and 9 a.m. next morning. Samples of blood were removed at noon each day and after the second sample was obtained apomorphine was again given to empty the

TABLE IV.

*Effect of Repeated Subcutaneous Injections of 1 Mg. per Kilo of Body Weight of Apomorphine on the Amount of Chlorine Lost in the Gastric Secretion; Also Effect on Percentage of Corpuscles in the Blood and Percentage of Protein in the Blood Serum.*

Dog No.	Weight of dog.	Plasma chlorides before.	Plasma chlorides after.	Total chlorine in vomitus.	Chlorine in vomitus per lb. of body weight.	Corpuscles in blood before.	Corpuscles in blood after.	Serum protein before.	Serum protein after.
		lbs. mg. Cl per 100 cc.	mg. Cl per 100 cc.	mg.	mg.	per cent	per cent	per cent	per cent
25	20	395	355	880	44.0	56.7	54.2	6.34	6.12
28	18	390	380	840	46.6	56.7	48.0	6.68	7.83
29	20	380		330	16.5	67.0	69.0		
30	17	385	380	310	18.2	71.1	63.0		
39	13	400	385	318	24.4	42.4	35.2	5.47	5.68
40	16	390	385	973	60.0	37.7	33.8	6.34	6.77
41	15	375	375	724	48.0	51.8	32.6	4.60	5.47
42	16	380	380	673	42.0	54.6	35.1	7.20	7.76
43	15	385	375	368	24.4	55.0	37.4	5.68	5.36
44	15	385	400	275	18.3	66.6	42.6	6.98	6.55
Average.....		386	382		34.2	55.9	45.0	6.16	6.44

stomach. The vomiting was usually quite copious. The amount of chlorine in the vomitus was estimated. Plasma chlorides were determined in the samples of blood, also the percentage of corpuscles and the serum protein. The results are given in Table IV.

A comparison of Tables III and IV is of interest. It is seen that practically no reduction of plasma chlorides occurred after the

administration of apomorphine although the amount of chlorine lost in the vomitus was even greater than after the administration of histamine. Objection may be taken to the inclusion of Dogs 37 and 38 in Table III as these dogs evidently did not react to histamine in the same manner as the other six animals. Even if these two dogs are omitted from the table the average values are as follows: plasma chlorides before the administration of histamine 382 mg. per 100 cc., plasma chlorides after the administration of histamine 345 mg. per 100 cc., average chlorine lost in the vomitus 37.9 mg. per pound of body weight. This last value is only slightly greater than the average amount lost in the vomitus after the use of apomorphine. It is evident that the reduction of blood chlorides bears no relation to the amount of chlorine lost in the vomitus.

It has been shown by Dale and Laidlaw (4) that the *intravenous* administration of histamine causes a dilatation of the capillaries. This allows the plasma to pass out of the capillaries and results in an increased concentration of the blood. From the figures reported in Table III it is seen that the percentage of corpuscles in the blood is actually decreased after the subcutaneous administration of histamine. On the other hand there is a slight increase in the percentage of serum protein which indicates an increase in the concentration of the serum. These same changes are also present however after the administration of apomorphine (Table IV) so it cannot be concluded that any specific action of histamine is responsible for the decrease in the percentage of corpuscles or the increase in the percentage of serum protein.

#### CONCLUSIONS.

1. The repeated subcutaneous injection of large amounts of histamine (ergamine acid phosphate) results in a definite reduction of the plasma chlorides of dogs. This reduction does not appear until the intoxication has been present for some hours.
2. The degree of intoxication apparently bears no relation to the decrease in the concentration of the plasma chlorides.
3. The reduction of the concentration of blood chlorides after the subcutaneous administration of histamine is not the result of a loss of chlorine in the gastric secretion.

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## THE EFFECT OF HIGH PROTEIN DIETS ON THE KIDNEYS OF RATS.

By HENRY JACKSON, JR., AND MARGARET D. RIGGS.

*(From the Thorndike Memorial Laboratory, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.)*

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In recent years much interest has attached to the rôle of high protein diets in the production of chronic nephritis. In 1919 Newburgh (1) fed egg white, casein, and soy beans to three different groups of rabbits over relatively long periods of time and found evidence of renal injury of an acute, subacute, or chronic nature in a majority of his animals. Many of them, however, showed varying degrees of malnutrition and the pathological changes were not strictly those of chronic nephritis as seen in man.

In 1921 Squier and Newburgh (2) gave high protein diets to cases with essential hypertension and found that after varying periods of time (2 to 21 days) red cells and albumin appeared in the urine which had previously been free from these abnormalities. At the same time the blood urea rose. It is noteworthy, however, that in three of the five experiments, salt was added to the diet just prior to the appearance of the urinary abnormalities (thus introducing a complicating factor of unknown importance), and it is further noteworthy that the blood urea nitrogen tended to regain its previous level before the period of high protein feeding was over, indicating that the effects of the high protein diet might well have been temporary rather than permanent. In the same paper are described experiments on normal individuals who ate 1 to 1½ pounds of steak at one meal. Albumin did not appear as the result of this test but red cells were noted in all cases after the meal. But because a single large dose of a given substance produces a certain pathological result it does not by any means follow that smaller doses over a long period of time will produce the same result.

In 1923 Newburgh and Clarkson (3) described dilatation of the tubules and some scarring of the glomeruli in rabbits after diets of 27 to 36 per cent protein for periods of from 6 to 12 months. Again in 1923 Polvogt, McCollum, and Simmonds (4) kept rats on diets containing 31 to 40 per cent protein for periods of from 100 to 480 days. Growth was normal. Chronic nephritis was described as the result, but comparatively little pathological or clinical evidence is brought forward to support the conclusion.

Finally Osborne and Mendel (5) fed rats on extremely high protein (40 to 80 per cent) diets for from 3 to 14 months. Growth was normal. The gross and microscopical examination of the kidneys failed to reveal any changes of an inflammatory, degenerative, or proliferative nature, although the kidneys were nearly double the normal weight. .

The evidence, therefore, that has so far been brought forward is of a somewhat conflicting nature. Rabbits are not ideal animals for such experimentation. They are normally herbivorous and are subject in varying degree to naturally occurring nephritis. Rats on the other hand are almost invariably free from spontaneous renal lesions and are normally omnivorous. Further, they are easily controlled, and Donaldson and others have supplied well established data for the normal state. It may be objected that rats are, perhaps, not susceptible to chronic nephritis and are not, therefore, any more suitable for such experiments than rabbits. But if in such a relatively immune animal pathological changes can be produced, the evidence is doubly important; and the mere rarity of spontaneous nephritis in rats in no way militates against the possibility of producing renal disease in them by appropriate means. Further, it should be understood that we do not consider our results with rats to be a repetition for purposes of confirmation or rejection of the results of Newburgh on rabbits; we believe, merely, that they may aid somewhat in solving the general problem of the relation of high protein diets to chronic nephritis.

With a view to further clarifying this important and interesting problem, a series of rats of known ancestry and pure, healthy stock was fed various diets for periods approximating a third of the animal's life.

The standard diet was made up of pure casein 20 per cent, pure arrowroot starch 56 per cent, creamery butter thoroughly washed

with water 15 per cent, salt mixture 4 per cent, and dried yeast 5 per cent.

Experimental Diet 1 was identical with the standard diet except that the casein was 76 per cent and there was no starch.

Experimental Diet 1a was the same as experimental Diet 1 except that there was added enough sodium bicarbonate to neutralize the sulfuric and phosphoric acids formed by the oxidation of the casein, thus preventing any disturbing factor of additional acidity in the diet.

In experimental Diet 2 pure egg albumin (Merck) was substituted for the starch. Otherwise, it was the same as the standard diet. Thus it contained 20 per cent casein and 56 per cent egg albumin or 76 per cent protein in all.

All rats were given water freely and each was confined to a cage by itself. All rats were given a small amount of fresh cabbage or carrot twice a week. Food was given to each animal each day slightly in excess of what he would actually eat. The amount actually eaten was carefully estimated each day. It was remarkable with what accuracy and uniformity the various rats ate. Those upon standard diet consumed, on the average, 0.99 gm. of protein per 100 gm. of body weight per day. Those on experimental Diets 1 and 1a (high protein and high protein plus alkali) consumed 3.90 gm. of protein per 100 gm. rat per day, and those on egg albumin ate 3.60 gm. Figured in calories this shows that the controls ate 21.8 calories per 100 gm. rat per day, those on high protein 22.8 calories, and the high egg albumin series 20.7 calories. In this connection it should be remembered that the rats varied greatly in weight and had always before them more food than they would eat. The figures become even more striking if the young growing rats are omitted, since they eat for a time very much more heavily than the adults. It is seen, therefore, that the high protein and albumin diet series consumed four times as much protein as the controls and that all diets were adequate calorically and practically efficient.

All rats on all diets did well, with the exception of one rat on high albumin diet which acquired a skin lesion and was killed. This rat is not included in the series reported.

The controls (three in number) averaged 260 gm. at the beginning of the experiment; at the end they averaged 298 gm. The



adult rats on high protein diet (three in number) averaged 220 gm. at the beginning and 265 at the end of the experiment. The young rats on this diet (two in number) averaged 54 gm. at the start of the feeding and 305 at the end. The rats on high proteins plus alkali (three in number) averaged 240 gm. at the start and 260 at the end. The rats on egg albumin (two in number) averaged 74 gm. at the start and gained to an average of 284 gm.

The rate of growth in each case was steady except during the hot month of August, when there was a loss of from 5 to 15 gm. in each rat, including the controls. Otherwise, the young rats gained rapidly and consistently and the old ones continued to gain slowly and became very fat.

During the progress of the experiment each rat was periodically placed in a metabolism cage and the urine collected and analyzed. All rats, so far as our experience goes, show slight albuminuria, the amount in each case being characteristic and uniform for that animal—usually from 2 to 20 mg. per day. Normal rats do not show casts or red cells.

In none of our rats did the albumin excreted increase during the period of the experiment, nor did the rats on experimental diets show more albumin than those on the standard diet. There was no evidence of nitrogen retention to be found in the urinary analyses. The amount excreted corresponded to the intake. Attempts were made to estimate the amount of amino acid excreted. Experience convinced us, however, that this could not be done with sufficient accuracy to warrant publication. It may be said, however, that on standard diet the amino acid nitrogen is about 1.5 per cent of the total. It does not increase proportionately with the total nitrogen; if the latter is quadrupled the amino acid nitrogen is not more than doubled.

Casts were not found in any of the specimens except rarely in one of the rats (No. 9) on high protein plus alkali. Red cells were never seen.

At the conclusion of the experiment each animal was etherized and the throat cut. The blood was collected and the non-protein nitrogen determined. The non-protein nitrogen of the control rats averaged 36.8 mg. per 100 ml. of whole blood; in the case of the high protein diets the average was 51.1. This is what is to be expected and is not in our opinion an indication of renal damage.

In general the non-protein nitrogen level of healthy individuals is proportional to the protein intake at the time. Further, it is extremely unlikely that the kidneys of all the high protein rats would have been injured with such complete uniformity with respect to their ability to excrete nitrogen.

Red counts were done shortly after the beginning of the experiments and again at the time of death. In no case did an anemia develop. All the red counts were between 8 and 9 million. The red cells appeared normal. In no rat of this series did the kidney lose its power to concentrate nitrogen. The marked diuresis in all the high protein rats was very striking and was of such an order as to keep the nitrogen concentration at about 4.5 gm. nitrogen per 100 ml.—exactly as on standard diet. The fairly constant pH values indicate that no marked disturbance of the neutrality mechanism was brought about by feeding excess acid-producing protein.

From a clinical point of view, therefore, we were unable to find any evidence of chronic nephritis.

The rats were autopsied and each tissue was studied microscopically by means of paraffin sections stained with eosin-methylene blue. In no case did we find either in the tubules or glomeruli or blood vessels of the kidneys any evidence of inflammatory, degenerative, or proliferative lesions. The kidneys of all rats appeared microscopically and grossly normal.

The kidneys of rats on high protein diets (Nos. 1, 1a, and 2) were all considerably hypertrophied, on the average 23.6 per cent heavier than the normal for the size rat (Donaldson (6)). There was less hypertrophy than other authors have found in similar studies, and this may in part be explained by the fact that all our rats were unusually fat and may have been "overweight," as the control kidneys were 5 per cent underweight according to Donaldson's figures.

All other organs were normal except in one control and one high protein animal in both of which there was found a curious pseudotuberculous lesion in the lungs. There was no evidence of arteriosclerosis.

There was, in short, no evidence obtained from pathological examination that the kidneys had been injured.

The table shows the average figures computed for the entire

TABLE I.

Rat No.	Sex.	Diet.	Age at start.		Age at finish.		Weight at start.	Weight at finish.	Months on diet.	Calories per 100 gm. per day.		Urine volume per 24 hrs.	Al- bumin per 24 hrs.	pH	Daily N intake.		Daily N per 100 gm.		Urine N daily.	Non-protein blood.		Weight of kidneys.	Per cent hypertrophy.
			mos.	mos.	mos.	mos.	gm.	gm.		gm.	gm.	cc.	mg.		gm.	gm.	gm.	gm.	gm.	mg.	gm.		
1	♂	Standard.	6	20	6	20	365	421	14	21.9	21.9	10.5	21.2	6.3	0.625	0.159	0.491	0.159	0.491	40.1	40.1	2.0	± 0
2	♀	"	6	24	6	24	207	233	18	20.1	20.1	6.5	4.8	6.3	0.321	0.146	0.290	0.146	0.290	36.1	36.1	1.7	-10
3	♀	"	6	16	6	16	207	240	10	23.8	23.8	8.5	11.6	6.0	0.388	0.173	0.377	0.173	0.377	34.2	34.2	2.8	+16.6
4	♂	1	6	20	6	20	250	294	14	20.5	20.5	29.5	7.1	5.8	1.54	0.568	1.32	0.568	1.32	60.1	60.1	2.7	+28.5
5	♀	1	6	20	6	20	216	256	14	20.3	20.3	25.5	16.2	5.9	1.33	0.560	1.12	0.560	1.12	50.2	50.2	2.7	+35
6	♀	1	6	19	6	19	195	246	13	20.0	20.0	23.0	22.4	6.1	1.21	0.558	1.16	0.558	1.16	52.5	52.5	2.8	+16.5
10	♂	1	1	16	1	16	48	305	15	25.8	25.8	29.0	14.4	6.2	1.26	0.712	1.15	0.712	1.15	58.1	58.1	2.4	+33
12	♀	1	1	21	1	21	61	205	20	33.8	33.8	24.0	12.4	5.9	1.23	0.925	1.15	0.925	1.15	52.6	52.6	3.8	+46
7	♂	1a	6	19	6	19	305	324	13	20.0	20.0	29.5	12.2	6.3	1.72	0.549	1.45	0.549	1.45	56.3	56.3	2.4	+26
8	♀	1a	6	19	6	19	196	226	13	21.2	21.2	24.0	14.8	6.4	1.27	0.586	1.10	0.586	1.10	47.2	47.2	2.4	+26
9	♀	1a	6	20	6	20	220	230	14	21.4	21.4	25.0	19.1	6.0	1.36	0.592	1.09	0.592	1.09	48.3	48.3	2.6	+8.3
14	♀	2	1	13	1	13	83	296	12	21.8	21.8	22.0	4.2	5.8	1.14	0.504	1.00	0.504	1.00	43.3	43.3	2.2	± 0
15	♀	2	1	16	1	16	66	273	15	19.8	19.8	20.0	18.2	6.1	0.93	0.546	0.90	0.546	0.90	43.3	43.3	2.2	± 0

period for each rat. The calories figured per 100 gm. rat per 24 hours were based on the mean weight and the actual amount of food eaten over the entire period, each day's intake being calculated. In the column "Urine volume per 24 hrs." is given the average urine volume in cc. as actually determined for three 4 day periods at intervals throughout the experiment. The albumin is given as the average daily figure determined by phosphotungstic acid and centrifugalization. The pH was determined colorimetrically. The daily nitrogen intake is computed from the food actually eaten and given as total gm. of nitrogen per 24 hours. The column headed "Daily N per 100 gm." gives the average daily nitrogen intake per 100 gm. of body weight. The urinary nitrogen is based on average figures for three 4 day periods at intervals throughout the experiment and gives total nitrogen output per 24 hours. It is to be noted that the actual figures for the urinary nitrogen fall short of the calculated nitrogen intake. This discrepancy is to be explained partly by the fact that a certain amount of urine was lost in the metabolism cages, and partly by the fact that the rats ate somewhat less in these cages than they did in the open cages in the animal house. The non-protein nitrogen is given in mg. per 100 ml. of whole blood and was determined by Folin's method. The blood was taken after primary anesthesia immediately before autopsy. The percentage hypertrophy is the amount of increase of weight over the standard normal figure given by Donaldson.

#### CONCLUSION.

By feeding very high protein diets over a period of from 10 to 20 months or about a third of a rat's life, we have been unable to produce in these animals any recognizable nephritis.

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# THE SITE OF AMMONIA FORMATION AND THE PROMINENT RÔLE OF VOMITING IN AMMONIA ELIMINATION.

By SIDNEY BLISS.

*(From the Biochemical Laboratory, Harvard Medical School, Boston.)*

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## INTRODUCTION.

The literature dealing with ammonia as related to metabolic processes contains an immense amount of data. Such aspects of the subject as pertain to the site of ammonia formation are adequately reviewed up to 1921 by Nash and Benedict (1). While the contributions published prior to the work of Folin and Denis (2, 3) in 1912 serve as the groundwork for later and more exact ammonia determinations (particularly in blood), it is unquestionably true that present day conceptions must be based chiefly upon the work that has been done since that date.

The conclusion of Nash and Benedict (1) relative to ammonia formation, namely that it has an exclusively renal origin, has found rather general confirmation and acceptance in the contributions that have appeared upon the subject since their publication in 1921 (4, 5, 6, 7, 8, 9). It has seemed to us that their unique hypothesis of the renal origin of ammonia rests upon experimental data that does not include the study of any other individual organs than the kidneys, and merits a more extensive investigation of the subject from this standpoint before one can be fully convinced that so unusual and specialized a function is indeed that of the kidney alone.

In brief, they found the renal vein to contain blood with two to three times the concentration of ammonia found in arterial blood. They assumed that the blood of the inferior vena cava below the entrance of the renals would reflect in its ammonia content the production of this substance elsewhere in the body, and on finding

but slight variations from arterial values in such samples they concluded that the kidney alone possessed this special function. Their conclusive argument hinged upon the results they obtained with bilateral nephrectomies and ureter ligations in dogs. With urinary ammonia elimination thus excluded, they reasoned that not only was the animal rendered incapable of excreting non-volatile acids, but that under such conditions there was more than the normal stimulus to ammonia production by other organs. Further, if any other organ proved capable of such ammonia formation to neutralize the accumulating acids, there would be an accumulation of ammonia in the blood from which it could not be excreted. Their results indicated to them that *ammonia did not accumulate* in the blood under these conditions, and thus it was shown that it was not being formed elsewhere than in the kidneys.

A detailed study of the problem has led us to modify this view as to the site of ammonia formation even though our experimental findings are in quite close agreement with those published by Nash and Benedict.

Since the work of Folin and Denis (2, 3) there have been remarkably few contributions to the literature that carry conviction as to the flawlessness of the technique employed to determine ammonia in blood. A notable exception is the work of Nash and Benedict (1). They used the Folin-Denis method in principle, and by the introduction of minor modifications, largely matters of individual preference, developed a satisfactory technique. Their results tend to confirm the earlier low values reported by Folin and Denis (2).

The micro titration method of Barnett (10) gave low values for normal human blood. Like all other titration methods it is open to the criticisms made by Matthews and Miller (11), namely that ammonia forms hydrolyzed salts yielding an indefinite end-point, “. . . the limits of error being several times greater than the amount of ammonia to be determined.”

In 1922 Gad-Andresen published a blood ammonia method (12) but admits a possible error greater than the variations we have found under a great variety of experimental conditions.

Bisgaard and Nørvig (13) reported that in checking up their formerly published results (14), obtained with the titration method of Henriques and Christiansen (15), they were unable to recover the ammonia from blood in less than 3 hours aeration. They attributed the low values of Nash and Benedict to incomplete aeration and found that they got values comparable to the low ones when they, too, used the shorter period of aeration (less than 30 minutes). In a later communication (16) they take the same

stand. Both the published results of Nash and Benedict (Table V) and those to be presented here show that such suspicions are without foundation.

The results reported by Russell (7) are of the same order as the low ones obtained by Folin and by Nash and Benedict. Matthews and Miller (11) call attention to the fact that their high values of 0.42 mg. were obtained with dogs, while Folin worked with cats. That the differences are not wholly ones of species is amply demonstrated by the later work of Nash and Benedict and the present one.

Henriques and his coworkers have made numerous contributions to the subject. In 1917 Henriques and Christiansen (15) published a titration method, and using it in a later communication (17) they were unable to confirm the results of Folin and Denis who had previously shown that portal blood was much richer in ammonia than systemic blood. So far as the writer is aware, this finding is without a parallel in the literature. In 1923, by the use of the same method, Henriques (18) contradicted the findings of Nash and Benedict, being unable to detect any differences in ammonia content between arterial and venous blood of the kidney. This seems to be the only finding of this sort on a carnivorous animal. The latest article that we have seen, Henriques and Gottlieb (19), 1924, states that, after all, *there is no ammonia in blood*, and for that reason Nash and Benedict must be right about the renal origin of ammonia, for under these circumstances any other site is automatically eliminated. These final and revolutionary results are now obtained with a newly devised method that is again a *titration* method.

#### EXPERIMENTAL.

##### *Technique.*

The method used was essentially the same as the one devised by Folin and Denis and modified slightly by Nash and Benedict. It is the opinion of the author that this method should not be attempted in any other connection than under rigid research conditions where a great amount of time and effort may be devoted to obtaining a single result. Only by the utmost precaution at all points in the procedure can one expect to get absolute and relative values that have any significance. Ammonia-free potassium oxalate was prepared according to the directions of Folin (20) by boiling a 5 per cent solution of the salt with potassium hydroxide, and neutralizing with oxalic acid. The salt was then recrystallized from ammonia-free water. Sodium carbonate was ignited to remove any organic impurities and our blanks showed it to be ammonia-free. Distillation from dilute sulfuric acid (an all glass apparatus was made for this) yielded water that was



ammonia-free. Hydrochloric acid for use in the acid collection tube during aeration was prepared ammonia-free by dilution of the concentrated acid from a freshly opened bottle. Caprylic alcohol was used to prevent frothing. Because we aerated for from 20 to 30 minutes instead of the 10 minute period of Nash and Benedict<sup>1</sup> correspondingly more caprylic alcohol was necessary. In order to prevent the formation of a precipitate with the caprylic alcohol on Nesslerizing, the acid collection tube was aerated separately before adding Nessler solution. The contents of the acid collection tube (3 cc. of water and 3 drops of acid) were washed into a glass-stoppered 10 cc. volumetric flask, and the flask filled nearly to the mark. 0.1 cc. of undiluted Nessler solution was added to the flask, and it was then filled to the mark. Colorimetric comparison was made with the colorimeter modified

TABLE I.  
*Recovery of Ammonia Added to Water.*

NH <sub>3</sub> -N per 100 cc.			
Added.	Recovered.	Added.	Recovered.
mg.	mg.	mg.	mg.
0.05	0.06	0.20	0.20
0.05	0.05	0.20	0.20
0.10	0.10	0.40	0.39
0.20	0.18	0.40	0.38
0.20	0.17	0.41	0.40

as described by Folin and Denis—the unknown on one side in a calibrated 100 mm. polariscope tube, and the standard in the regular colorimeter cup, but provided with an iris diaphragm for the adjustment of the intensity of light equally on the two sides.

Our reagents were controlled by running complete determinations with water in place of the sample, and when these controls were absolutely negative or gave values less than 0.005 mg. NH<sub>3</sub>-N per 100 cc., known amounts of ammonia, as the sulfate, were next added to water—and quantitatively recovered. Table I gives a representative portion of the results.

*Criterion of Accuracy of the Technique.*—Working with a uniform

<sup>1</sup> We place no particular emphasis on this difference in aeration time.

technique as described, when 0.20 mg. of ammonia-nitrogen as ammonium sulfate was added to water, 0.196 mg. was recovered with a standard deviation of  $\pm 0.019$ . The coefficient of variation is, then, approximately 10 per cent. Therefore, variations noted in the experiments recorded beyond this limit of accuracy must be regarded as significant.

Table II shows the quantitative recovery of ammonia added to blood.

*Technique of Taking Samples of Venous Blood.*

The often mentioned precaution of pointing a needle toward the organ from which a sample of venous blood is to be taken is not a certain means of getting the desired sample. With the bevel of the needle turned toward the pancreas the desired sample is probable. With the needle rotated 180 degrees we encounter the

TABLE II.  
*Recovery of Ammonia Added to Blood.*

NH <sub>3</sub> -N per 100 cc. of blood.			
Original.	Added.	Recovered.	Error.
mg.	mg.	mg.	mg.
0.10	0.20	0.29	0.01
0.19	0.10	0.29	0.00
0.22	0.40	0.61	0.01
0.05	0.20	0.24	0.01

possibility of getting blood from the portal vein as well as the pancreas. The ratio of the caliber of the needle to the size of the vein is, of course, a factor here. In cases where the two are of nearly the same size this distinction becomes relatively more important. The most important precaution, and one that admits of direct control by observation, is that of making sure that the vein in question does not decrease in diameter during the time that the sample is being withdrawn. With no perceptible change in diameter of the vein and no interference with blood flow through the organ, blood is being drawn from a constant stream leaving the organ. No stasis comes in as a possible complication.

*Examination of the Various Organs.**A. Carnivorous Animal (Dog).<sup>2</sup>*

A large series of ammonia determinations was performed on arterial and venous blood from the kidney, pancreas, spleen,

TABLE III.

*Comparison of the Ammonia Content of Arterial and Venous Blood in the Dog.*

*NH<sub>3</sub>-N per 100 Cc. of Blood. Immediate Aeration.*

*Carotid Artery versus Renal Vein.*

Dog No.	Carotid.	Renal vein.	Remarks.
Non-nephritic dogs.			
	<i>mg.</i>	<i>mg.</i>	
11	0.10	0.21	Normal; mangle; fasting.
12	0.09	0.30	" acid feeding; † in digestion.
14	0.09	0.28	" " " † fasting.
3	0.07*	0.26	Phlorhizin; fasting.
4	0.10	0.21	" "
13	0.09	0.20	Tetany of thyroparathyroidectomy.
21	0.14	0.14	Normal; in digestion.
22	0.11	0.18	" albuminuria; in digestion.
27	0.21†	0.23	" in digestion.
28	0.09†	0.26	" " "
33	0.10†	0.23	Ligated ureters; non-protein N 111.
Nephritic dogs.			
7	0.06	0.36	Uranium poisoning; fasting.
8	0.13	0.21	" " " non-protein N 314.
8	0.15†	0.36	" " 45 min. after HCl injection.
10	0.15	0.30	Uranium poisoning; non-protein N 571.

\* Renal artery.

† Femoral artery.

femorals, and the head (carotid-jugular) as well as a smaller number of isolated determinations (thyroid, adrenals).

Primary interest attached to the values obtained for the kidney. Table III gives abundant confirmation of the values reported for

\* My thanks are due Mr. Henry Martin for much timely help in the management of animals.

the first time by Nash and Benedict; namely, two to three times the quantity of ammonia in venous blood as compared with arterial.

Our arterial and renal vein values compare closely with those reported by Nash and Benedict. Table III gives a comparison

TABLE IV.

*Comparison of the Ammonia Content of Arterial and Venous Blood in the Dog-  
NH<sub>3</sub>-N per 100 Cc. of Blood. Immediate Aerations.  
Carotid Artery versus Pancreaticoduodenal Vein.*

Dog No.	Carotid.	Pan- creatico- duodenal vein.	Remarks.
Non-nephritic dogs.			
9	0.06	0.20	Normal; in digestion.
11	0.10	0.31	" mangle; fasting.
12	0.09	0.29	" acid feeding; in digestion.
14	0.09	0.28	" " " fasting.
4	0.10	0.12	Phlorhizin; fasting.
13	0.09	0.17	Tetany of thyroparathyroidectomy.
21	0.14	0.18	Normal; in digestion.
22	0.11	0.15	" " " albuminuria.
25	0.05	0.26	" double kidney ligation and 30 cc. N HCl injected.
27	0.21*	0.36	One kidney ligated; in digestion.
28	0.09*	0.24	Normal; in digestion.
33	0.10*	0.17	" ligated ureters; non-protein N 111.
Nephritic dogs.			
7	0.06	0.31	Uranium poisoning; fasting.
8	0.13	0.21	" " " non-protein N 314.
8	0.15	0.36	Same 35 min. after HCl injection.
10	0.15	0.29	Uranium poisoning; fasting; non-protein N 571.

\* Femoral artery.

of these values. It is seen that in the normal, non-nephritic dog, the renal vein contains from two to three times the ammonia of the arterial circulation. The values here, as well as through all the other experiments, are higher for the nephritic dogs than the others.

That another organ shares equally with the kidney in ammonia-

yielding capacity is shown by Table IV which compares arterial values with venous blood from the pancreaticoduodenal vein. The average values for the non-nephritics (excluding kidney ligations and ureter ligations) are 0.10 mg. and 0.21 mg. for the pancreaticoduodenal vein as compared with 0.11 mg. and 0.23 mg. for the renal vein. Again, both arterial and venous values are higher in the nephritic dogs.

Because of its anatomical relationships to the pancreas and the duodenum, the pancreaticoduodenal vein is of special interest. To be sure that we were not getting portal blood, when drawing from the pancreaticoduodenal vein the expedient was tried of clamping it at its entrance into the portal. This did not prevent our getting the high values for this vein (Dog 12). In several experiments (Dogs 7, 9, and 10) the values for the pancreaticoduodenal vein were higher than the portal blood value—and this would obviously have been impossible had the taking of the sample been at fault. Values for portal blood were usually obtained, but they add nothing to what is already known of portal ammonia. A few of the values were remarkable for their low ammonia content, and tend to substantiate the view expressed by Folin and Denis (2) that high portal values are connected with decomposition in the intestine rather than a production of ammonia by the walls of the gut. Verification at autopsy of the fact that the duodenum was clean, as well as the occasional low values for portal blood when decomposition in the intestine was decreased, together with the fact that the gastric branch of the gastrosplenic vein did not alter the ammonia value of the venous return from the spleen itself—all of these facts assured us that the high values for the pancreaticoduodenal vein were due to the ammonia produced by the pancreas and not ammonia absorbed from, or produced by, the walls of the gut.

A survey of Table V indicates that the differences found for the other organs are reflected in those for the spleen. The average non-nephritic values here are 0.10 mg. and 0.13 mg. and the nephritics show values of 0.11 and 0.18 mg. The same regularity is seen among the nephritics themselves.

It would not be expected that a small gland like the adrenal, with a central portion of nerve-like tissue, would form appreciable amounts of ammonia, and this has been found to accord with the

facts. It seemed worth while to make a few analyses of blood entering and leaving the liver, and according to expectations the blood of the hepatic vein had an ammonia content comparable to the systemic values. In Dog 22 we found the blood of the portal vein to contain 0.20 mg. of ammonia nitrogen and of the hepatic

TABLE V.

*Comparison of the Ammonia Content of Arterial and Venous Blood in the Dog.  
NH<sub>3</sub>-N per 100 Cc. of Blood. Immediate Aerations.  
Carotid Artery versus Splenic Vein.*

Dog No.	Carotid.	Splenic.	Remarks.
Non-nephritic dogs.			
9	0.06	0.10	Normal; in digestion.
11	0.10	0.15	" mangle; fasting.
12	0.09	0.25	" acid feeding; in digestion.
14	0.07*	0.09	" " " fasting.
3	0.11†	0.15	Phlorhizin; fasting.
4	0.10	0.08	" "
4	0.11	0.11	Same; both kidneys clamped 69 min.
13	0.09	0.12	Tetany of thyroparathyroidectomy.
19	0.08	0.09	Normal; fasting.
21	0.14	0.14	" in digestion.
22	0.11	0.11	" " " albuminuria.
25	0.05	0.12	Both kidneys ligated; 30 cc. N HCl injected.
28	0.09*	0.24	" " " in digestion.
Nephritic dogs.			
7	0.06	0.11	Uranium poisoning; fasting.
8	0.13	0.11	" " " non-protein N 314.
10	0.15	0.31	Uranium poisoning; fasting; non-protein N 571.

\* Femoral artery.

† Splenic artery.

vein 0.07 mg. In Dog 12 the respective values were 0.17 mg. and 0.10 mg. The thyroid vein we found to be low in ammonia. Two analyses of gall bladder bile gave values of 0.17 mg. and 0.46 mg. per 100 cc. of bile.

Table VI is a comparison of carotid with jugular blood. Normal dogs show no difference between the ammonia content of carotid

and jugular blood. Our averages are 0.10 mg. for each. Nephritic dogs present a different picture. All our averages for arterial and venous blood of nephritic dogs are higher than our averages

TABLE VI.

*Comparison of the Ammonia Content of Arterial and Venous Blood in the Dog.  
NH<sub>3</sub>-N per 100 Cc. of Blood. Immediate Aerations.  
Carotid Artery versus Jugular Vein.*

Dog No.	Carotid.	Jugular.	Remarks.
Non-nephritic dogs.			
	mg.	mg.	
5	0.10	0.11	Normal; chloralose; fasting.
9	0.06	0.06	" in digestion.
11	0.10	0.10	" mange; fasting.
12	0.09	0.10	" acid feeding;* in digestion.
14	0.09	0.10	" " " * fasting.
4	0.10	0.08	Phlorhizin; fasting.
4	0.11	0.10	Same 45 min. after clamping two kidneys.
13	0.09	0.11	Tetany after thyroparathyroidectomy.
19	0.08	0.10	Normal; fasting.
21	0.14	0.12	" in digestion.
22	0.11	0.09	" " " albuminuria.
25	0.05	0.06	Double kidney ligation; 30 cc. N HCl injected.
26	0.08	0.10	Two kidneys and pancreaticoduodenal vein ligated.
33		0.10	Ligated ureters; non-protein N 111.
Nephritic dogs.			
7	0.06†	0.11	Uranium poisoning; fasting.
8	0.13	0.16	" " " non-protein N 314.
8	0.15‡	0.16	Same 24 min. after HCl injection; non-protein N 314.
10	0.15	0.37	Uranium poisoning; fasting; non-protein N 571.

\* 700 cc. N/7 HCl by stomach tube.

† No non-protein on this dog; doubtful if this case is comparable with later severe cases.

‡ Femoral artery.

for arterial and venous blood of non-nephritics. Looking at the individual figures we find that not only are they higher than the non-nephritic figures, but among themselves they grade up as the

non-protein nitrogen values do—a suggestive correlation in the light of the rest of the data to be presented.

In Table VII we see that the normal values for arterial and venous blood from a purely muscular region average about what was found for carotid and jugular value (excluding Dog 27 which for unaccountable reasons showed very high values; the method was believed to be adequately checked before and after the experiment); namely, 0.11 mg. for the femoral artery and 0.10 mg. for the femoral vein.

TABLE VII.

*Comparison of the Ammonia Content of Arterial and Venous Blood in the Dog.  
NH<sub>3</sub>-N per 100 Cc. of Blood. Immediate Aerations.  
Femoral Artery versus Femoral Vein.*

Dog No.	Femoral artery.	Femoral vein.	Remarks.
Non-nephritic dogs.			
19	0.15	0.12	Normal; fasting.
21	0.16	0.13	" in digestion.
22	0.08	0.08	" albuminuria; in digestion.
23	0.09	0.07	" atrophic pancreas; in digestion.
26	0.13	0.14	" ligation of kidneys and pancreaticoduodenal vein; mange.
26	0.09	0.10	Same 2 hrs. later.
27	0.21	0.23	Normal; in digestion.
27	0.35	0.18	Same, kidneys tied 1 hr.
28	0.09	0.08	Normal; in digestion.
28	0.14		Same, kidneys tied 90 min.
33	0.10		Ligated ureters; non-protein N 111.

While the increase for the arterial value appears to be small, it should be noted that both Dogs 27 and 28 showed 60 per cent increases in arterial values after kidney ligation, without increases in venous values. It will be noticed that Dog 26, after kidney ligation showed a decrease in both arterial and venous values, but the pancreaticoduodenal vein was also ligated.

Nash and Benedict (p. 482) say, "The blood from the renal vein averages twice as much ammonia as does the blood from other sources. These differences are so marked as to admit of only one interpretation; *viz.*, that the kidney, instead of excreting ammonia from the blood, forms the ammonia which it excretes,



while at the same time it contributes a small amount of ammonia to the blood."

Accepting this view, our results from the other organs particularly the pancreas, compel us to believe that the kidney is *not* "the center of ammonia production in the body" in the sense intended by Nash and Benedict.

There is no justification in disregarding organs such as the pancreas and spleen. Even though their venous blood eventually joins the blood of the portal vein, rich in ammonia formed during intestinal putrefaction, the ammonia formed by the pancreas, if it is ammonia formed to neutralize mineral acids in the sense that the ammonia of the renal vein is ammonia formed to neutralize acids, cannot be considered as available for transformation into urea by the liver, but must be thought of as continuing on into the systemic blood stream paired with non-volatile acid. If the higher ammonia content of venous blood from the kidney admits of only one interpretation, namely ammonia formation by the kidney, the same must be said of the pancreas which gives identical values, and in varying degrees of other organs as well. Analyses of the blood from the inferior vena cava could not, of course, have revealed this situation.

It is also to be remembered that due to variations in blood flow through the organs, neither the results of Nash and Benedict nor the present ones can show the relative quantitative importance of the kidney and pancreas in terms of ammonia formation either gram for gram or organ for organ.

In their otherwise complete review of the literature, Nash and Benedict did not mention the part of the work of Horodyński, Salaskin, and Zaleski that most concerns the problem. These investigators not only report generally higher venous (21) than arterial values, but state, "Das Blut peripherischer Venen enthält mehr Ammoniak, als das Arterienblut, jedoch weniger als die das Pfortaderblut. Das Verhältniss des  $\text{NH}_3$ -Gehaltes in Arterienblut zu demjenigen des Blutes peripherischer Venen beträgt in Mittel 1:2."

Nash and Benedict found exactly this same relation between the ammonia content of arterial and venous blood in the kidney (a point entirely missed by Horodyński and his coworkers) and built around it their hypothesis of the exclusively renal origin of ammonia.

These results bring us face to face with the problem of explaining the failure of Nash and Benedict to observe any increases in the ammonia content of the systemic blood of doubly nephrectomized dogs. It would seem that they had produced the conditions which would demonstrate the ammonia-forming ability of the spleen and pancreas particularly. With entire confidence in their analytical results, which opinion we have never had to alter, we sought to duplicate their experiments and results.

Repetition of the experiments of Nash and Benedict upon doubly nephrectomized dogs and dogs with double ligation of the ureters yielded the same results as reported by them, *if certain conditions were fulfilled*. Ether anesthesia was used throughout.

TABLE VIII.

Double nephrectomy; bitch, weight 10.1 kilos, age 2.5 yrs.

Hrs. after operation.	Per 100 cc. blood.*		Vomit NH <sub>3</sub> -N.	Remarks.
	NH <sub>3</sub> -N	Non-protein N.		
	mg.	mg.	mg.	
Preliminary.	0.13			
0.5	0.22			Vomited meat.
1.0	0.18	74		" "
5.0	0.22	88		
6.0			18.2	" (clear).
6.25	0.18	95		
6.5			44.4	" "
7.5	0.18	97		
10.5	0.23	114		

\* All samples from the jugular vein.

Examination of Table VIII shows that after the first half hour value, there was no increase in the blood ammonia for the 10 hours that the experiment was conducted. It is significant that not only were there no increases during this 10 hour period, but that the blood ammonia values actually fell below the general level in samples taken after the dog had vomited.

Previous experience with doubly nephrectomized dogs led us to suspect that the invariable alternations of drinking and vomiting of such animals might furnish an explanation of the results of Nash and Benedict wherein they observed that blood ammonia values were not markedly increased.

In Table IX we see in more striking fashion the validity of this relationship between the level of ammonia in the blood and its

TABLE IX.

Dog 30, male; weight 5.75 kilos; age 18 months; 1½ kidneys extirpated.

Time.	Values per 100 cc. blood.*		Vomit.		Urine.		Remarks.
	NH <sub>3</sub> -N	Non-protein N.	Volume.	NH <sub>3</sub> -N	Volume.	NH <sub>3</sub> -N	
<i>1923</i>	<i>mg.</i>	<i>mg.</i>	<i>cc.</i>	<i>mg.</i>	<i>cc.</i>	<i>mg.</i>	
Oct. 20.....	0.09						Preliminary sample. Operation.
Oct. 21							
11.30 a.m.....			Lost.		6		Urine bloody.
12 m.....	0.11	250					
6.15 p.m.....	0.21	357					
Oct. 22							
9.00 a.m.....					22	4.4	
9.15 " .....			50	Lost.			
9.30 " .....			122	33.0			
11.30 " .....	0.22	391					
3.30 p.m.....	0.25						
3.30-5.30 p.m.			360	63.8			Hematocrit 30. 464 cc. water taken.
3.58 p.m.....	0.14				17	2.6	Hematocrit 31.
4.15 p.m.....							
Oct. 23, night specimen.....			380	56.7			665 cc. water taken.
12.17 p.m.....			110	36.6	17	1.1	
12.40 " .....	0.24	655					Hematocrit 25.
Totals.....			1022	240.1	62	8.1	

\* All samples from the jugular vein.

excretion by way of the vomitus when its urinary elimination is prevented. In this experiment enough kidney tissue was removed

to insure an acute renal death, yet prolonging the period sufficiently for extended observations on the relationships between blood ammonia levels and the elimination of ammonia by way of the vomitus.

Inspection of Table IX shows that Dog 30, in the 3 days following sub-total nephrectomy, voided but 62 cc. of urine containing, in all, only 8.1 mg. of ammonia nitrogen. In the same interval; 1022 cc. of vomitus carried with it an elimination of 240 mg. of ammonia nitrogen and not all the vomitus could be collected. That blood ammonia values are affected by the elimination of ammonia by way of the vomitus is again demonstrated in this experiment, when the striking reduction from 0.25 mg. to 0.14 mg. was made within a period of 20 minutes, coincident with the elimination of 63.8 mg. of ammonia nitrogen in the vomitus, vomiting being induced by forcing fluids by stomach tube.

The possibility was recognized that some of the ammonia of the vomitus might be formed secondarily from urea, and this might be particularly true of a dog having such high nitrogen retention. This point was controlled by introducing into the stomach of Dog 31 at different times, water and a 1 per cent urea solution in 5 per cent saline (NaCl as an emetic).

Dog 31; female; weight 3.6 kilos. This dog was fasted and given 865 cc. of water by stomach tube over a period of 33 minutes. 150 cc. of vomitus contained but 1.0 mg. of ammonia nitrogen. The same dog was given 300 cc. of a 1 per cent urea in 5 per cent sodium chloride solution, and 330 cc. were vomited and contained 0.55 mg. of ammonia nitrogen. On the following day the same dog was given 1530 cc. of the same strength urea and sodium chloride solution over a period of 110 minutes. 1270 cc. were vomited and again no ammonia was produced. These results are but a few of very many at widely varying time intervals that were necessary to overcome our own skepticism on the subject.

Double ureter ligation yielded results that were equally convincing. While Nash and Benedict state that under such conditions "ammonia cannot leave the organism," we are forced to the conclusion that results from such an experimental procedure cannot be correctly interpreted without an appreciation of the fact that the dog does possess in vomiting a means of eliminating ammonia.

Dog 33; white female poodle; age 1 year; weight 3.7 kilos. This dog was fasted 2 days before operation. On November 12, the dog was given ether

TABLE X.  
*Dog. 33. Ureter Ligation. The Role of Vomiting.*

Time.	Values per 100 cc. blood.		Water intake.	Vomit.		Remarks.
	NH <sub>2</sub> -N	Non-protein N.		Volume.	NH <sub>2</sub> -N	
	mg.	mg.	cc.	cc.	mg.	
Preliminary....	0.10	32				Measured water kept before the dog.
Half hr. ....	0.12		120	36	2.3	
1 hr.....	0.12		50	30	3.1	
2 hrs.....	0.12		120	20	1.3	
3 " .....	0.11		50	44	5.0	
4 " .....	0.09		60	Lost while taking blood.		
5 " .....	0.09		30	27	4.9	
6 " .....	0.09			No more vomitus for 17 hrs. Temperature 101°.		Temperature 102°.
Night.						
6 to 23 hrs....			210	None.		
23 hrs.....	0.19					
29 " .....		94	60	"		
30 to 31 hrs....			200			
			100			
			100	270	14.6	
			100			
			100			
31 hrs.....	0.08	98				
35 " .....			550	Not collected.		
36 " .....	Ether anesthesia.					
	0.10 femoral artery.					
	0.11 renal "					
	0.23 " vein.					
	0.17 Pancreaticoduodenal vein. Non-protein N 111.					

at 9.00 a.m.; at 9.10 the left ureter was ligated retroperitoneally; at 9.20 the right ureter was tied and a skin flap was made exposing the jugular; at

9.30 ether was discontinued; by 9.40 the dog was walking about the room. The peritoneal cavity was not entered, the dog suffered the very minimum of shock. This experiment was designed to determine the conditions under which double ureter ligation yielded no increases in ammonia of the blood, and also to find what relationship might exist between water intake, the vomitus, and the level of ammonia in the blood.

Table X shows very clearly why dogs with kidney function eliminated may furnish results that might, on first examination, tend to indicate that ammonia does not accumulate in blood. Samples taken 3, 4, 5, 6, 31, and 36 hours after operation are indeed normal. On careful analysis, however, it is unmistakable that only when the vomitus route of ammonia elimination is ruled out does accumulation occur. Blood ammonia values *do* increase with functionless kidneys, and they increase markedly as seen by reference to Table X. 23 hours after operation, when the fairly regular hourly elimination of 5 mg. of vomitus ammonia was stopped, the blood ammonia had doubled in value. Just following this accumulation, vomiting was induced by giving water by stomach tube, and after the elimination of 14.6 mg. of ammonia nitrogen the blood ammonia value was less than the preliminary sample taken before operation. These results confirm the *analyses* of Nash and Benedict on their dogs with renal impairment—and furnish, we believe, the correct interpretation of them. That ammonia formation was indeed going on is shown by the analyses made, for which the animal was sacrificed at the end of 36 hours.

#### *Rôle of Vomiting in Ammonia Elimination.*

Continued observations upon dogs with acute renal insufficiency led us to suspect that vomiting might play an important rôle in ammonia elimination. (Vomiting is very common in uremic patients.) Definite experiments, planned to cover the point, convinced us that herein lies the explanation of the results obtained by Nash and Benedict and ourselves.

To see if the vomiting reflex might have its origin in the stimulation of the gastric mucosa by the ammonium ion, we gave a normal dog 900 mg. of ammonia nitrogen as the carbonate by stomach tube—an amount many times the values we had encountered in our experiments, and it was entirely without effect.

On the other hand, our results show that changes which closely

parallel increases in the concentration of ammonia in circulating blood are accompanied by vomiting. These points were tested by the intravenous injection of ammonium carbonate, as well as by jack bean urease extracts. One experiment of each type is sufficient to illustrate the results we got in several experiments.

Dog 36; male; age 9 months; weight 6.55 kilos. With a preliminary blood ammonia value of 0.12 mg. the dog was given 2 cc. of an alcoholic extract of jack bean meal *via* the jugular vein; 7 and again 17 minutes later the dog vomited spontaneously. The blood ammonia value was found to be 1.04 mg., nearly nine times the preliminary value. At 35, 45, and 60 minute intervals after the injection the dog vomited spontaneously—and in all 15.8 mg. were eliminated.

In another experiment, an alcoholic urease solution was tried with a dog in moderately advanced uranium nephritis. This dog's blood ammonia value increased from 0.12 mg. to 0.75 mg., and 129.0 mg. of ammonia nitrogen were vomited.

#### *Injection of Ammonium Carbonate Solutions.*

Dog 31; female; age 9 months; weight 3.3 kilos. After preliminary trials on the previous day to determine dosage, the dog received 52.5 mg. of ammonia nitrogen as the carbonate. No change was noticed, and 15 minutes later 87.5 mg. were given. The dog appeared quiet and slightly uneasy. There was a bloody diarrhea. In 30 minutes after the first injection, 175 mg. additional were injected (all injections were into the jugular). The dog showed a partial loss of function of the hind limbs. At the end of 45 minutes 262.5 mg. were given and at once the typical convulsions ensued.

The blood ammonia value at the height of convulsions was 0.92 mg. By giving an emetic of 5 per cent NaCl we obtained 59 mg. of ammonia nitrogen in the vomitus. The dog made a complete recovery. This was the only dog that received these injections and failed to vomit spontaneously.

Dog 33; female; age 1 year; weight 3.8 kilos. Vomited spontaneously and repeatedly after two injections of 175 mg. each, and 43.2 mg. of ammonia nitrogen were so recovered. This dog also made a good recovery.

#### *Vomiting in Nephritis.*

The regular drinking and vomiting of our dogs poisoned with uranium (and also those with both kidneys removed) was a com-

mon observation. The data on one of them are sufficient to illustrate the amounts of ammonia so eliminated.

Dog 35; male; age 2.5 years; weight 20.7 kilos. On the 3rd day after the subcutaneous injection of 4 mg. of uranium nitrate per kilo, the dog had a blood non-protein nitrogen value of 272 mg. Anuria was nearly complete; the overnight specimen was but 5 cc. The dog was given 230 cc. of a 5 per cent NaCl solution, and in 4 minutes vomited 390 cc. of greenish-yellow, turbid material, very strongly acid, containing 82.1 mg. of ammonia nitrogen. Additional vomitus was not collected. Occasionally samples of vomitus were thus colored as if with bile, but more often they were entirely colorless. Our analyses of gall bladder bile show that the very low ammonia content of that fluid cannot explain such a result as this, even when bile is regurgitated into the stomach. The extremely clean character of the surface of the duodenum in these dogs which by this time have voluntarily fasted for days precludes all possibility of ammonia coming from this source even if it were not for the experiments on blood ammonia levels after vomiting, which demonstrate clearly the rôle of vomiting in these cases.

On the 4th day, with no urine, vomitus, or feces during the night, the dog passed only a few drops of urine. It was weak but walked about when encouraged to do so. The non-protein nitrogen value was 349. From 9 a.m. until 1.35 p.m. it was given 2200 cc. of water by stomach tube, and 1500 cc. of vomitus, acid in reaction, were collected, containing 300 mg. of ammonia nitrogen. At 1.40 p.m. the non-protein nitrogen value was again 349.

At 3.30 p.m. the dog suffered violent clonic spasms, the teeth chattered, limbs became rigid, pupils dilated, and respiration was labored. The symptoms died away, and the animal lapsed into a semiconscious stupor.

On the 5th day the dog could only stand when propped on his feet. The non-protein nitrogen value was 427 mg. Between 10.31 and 11.04 a.m. the dog was given 3000 cc. of water and 2900 cc. of vomitus were collected. The ammonia nitrogen content of the vomitus was 263 mg. At 11.10 a.m. the non-protein nitrogen value of the blood was again 427. The dog was found dead on the morning of the 6th day.

We believe that the evidence presented is sufficient to indicate that in vomiting the dog possesses a hitherto unsuspected means of elimination of considerable theoretical importance in the interpretation of results obtained especially with dogs with impaired kidney function.

#### *Significance of Arterial Values.*

It seems highly probable that most importance must, after all, be attached to arterial rather than venous levels of blood ammonia. Table VI gives the carotid values for a representative



portion of our dogs. In non-nephritics the values are generally lower, and in the nephritics (due to acidosis) higher. This would necessarily be the case if the ammonia of arterial blood is there on its way to the kidneys for excretion.

Starting with another purpose in view, we<sup>3</sup> gave Dog 66 (weight 11.1 kilos) small quantities of sodium bicarbonate by stomach tube at frequent intervals during the day and gave it a bone to eat during the night. In this manner we were able to insure a nearly constant plethora of inorganic fixed base together with a very high protein intake (meat). The day before operation the record was:

	<i>mg.</i>
Total urinary nitrogen.....	27,215
Ammonia nitrogen.....	89

$$\frac{\text{NH}_3 - \text{N}}{\text{Total N}} \text{ ratio} = \frac{1}{306}$$

Operating under amytal anesthesia we obtained the following values for the ammonia content of the blood:

Renal artery.....	0.02 mg. per 100 cc.
“ vein.....	0.08 “ “ 100 “

The urine was constantly alkaline for days previous to the operation, and complete ammonia analyses showed the dog to have consistent low ratios of ammonia nitrogen to total nitrogen. The urine secreted during the operative procedures was still alkaline, and we controlled the conditions under which we operated by the following analyses of the urine secreted during that time:

	<i>mg.</i>
Total urinary nitrogen.....	1322
Ammonia nitrogen.....	1.3

$$\frac{\text{NH}_3 - \text{N}}{\text{Total N}} \text{ ratio} = \frac{1}{1017}$$

There is no question but that here we have a complete picture of what happens to the concentrations of circulating ammonia when there is no chemical metabolic stimulus for its formation in the tissues. The arterial value found was the lowest one that we had then encountered in any carnivorous (or herbivorous) animal. If it is true that the kidney forms nearly all the ammonia for direct excretion, arterial levels could not thus reflect the effects of alkali administration. If, however, the various organs form

<sup>3</sup> My thanks are tendered Mr. Gustav Lindskog for his careful work in helping with this set of experiments.

ammonia which is later excreted *from arterial blood*, alkali administration should be reflected in a lowering of the level of ammonia in arterial and in renal venous blood. The above results show this to be the case, as do the following additional ones.

Dog 68 was kept on a *low protein diet* and *no alkali* was given. The daily values in the foreperiod were entirely consistent and regular, and on the day preceding the operation were:

Total urinary nitrogen.....	mg. 1763
Ammonia nitrogen.....	184
$\frac{\text{NH}_4\text{-N}}{\text{Total N}} \text{ ratio} = \frac{1}{9.5}$	

At operation the blood showed the following ammonia values:

Renal artery.....	0.08 mg. per 100 cc.
“ vein.....	0.19 “ “ 100 “

Though no attempt was made to lower the ammonia excretion—and the ratio of 9.5 is indeed very high—the actual amount (184 mg.) was relatively small for a 17 kilo dog, and was but the natural consequence of a diet low in protein. Here we see a moderately decreased call for ammonia to handle acids—and a slight decrease in the value for renal venous blood.

Dog 71. Kept on a *low protein diet with alkali* administration. Pre-operative day showed:

Total urinary nitrogen.....	mg. 3588
Ammonia nitrogen.....	14
$\frac{\text{NH}_4\text{-N}}{\text{Total N}} \text{ ratio} = \frac{1}{276}$	

And, at operation the blood ammonia values were:

Renal artery.....	0.026 mg. per 100 cc.
“ vein.....	0.129 “ “ 100 “

Here again we see the effects of alkali administration and very low ammonia excretion in (1) a *very low level of arterial ammonia* and (2) a *greatly reduced level of ammonia in venous blood*.

Further evidence may be adduced from the experiments of Nash and Benedict on alkali injection. They reported that dogs thus treated and showing an alkaline urine had lower levels of ammonia in renal venous blood than did their normals, and their values of 0.13, 0.16, and 0.18 mg. compare exceptionally well with ours of 0.08, 0.13, and 0.19 mg. per 100 cc. of blood. Their own

conclusion is that, "There is some evidence of an effect on the ammonia content of the blood of the renal vein, but further experiments would be required to warrant any definite conclusion here." It is perhaps almost needless to add that the low arterial and venous blood ammonia values in herbivorous animals are further confirmation of this view.

### *B. Herbivorous Animals (Rabbit, Goat).*

The goat and the rabbit offer us the chance of comparing herbivorous animals with the dog with reference to ammonia formation. These animals, living on diets of green grass, carrots, and cabbage have an alkaline urine of very low ammonia content. Three successive 24 hour collections from Goat 1 gave ammonia nitrogen values of 10.2, 11.2, and 12.8 mg. respectively (Table XI).

Each voiding of urine was collected from the animal directly while being led outside, or else the sample was collected from the cage as soon as voided. Fractional analyses were made immediately, so that the question of gain or loss of ammonia did not arise.

In two rabbits and one goat we were able to compare the ammonia content of the blood of the renal vein with that of arterial blood. Without exception we found the two values to be essentially identical, as contrasted with the marked differences obtained in the carnivora. One rabbit showed:

Renal artery.....	0.10 mg. $\text{NH}_3\text{-N}$ per 100 cc.
"    vein.....	0.08 " $\text{NH}_3\text{-N}$ " 100 "
and 40 minutes later, from the other kidney:	
Aorta.....	0.09 mg. $\text{NH}_3\text{-N}$ per 100 cc.
Renal vein.....	0.09 " " " 100 "

These particular absolute values are not offered as correct for the normal rabbit as this one gave birth to one young rabbit during the anesthesia and seven more were removed from the uterus. She was evidently just at term.

From another non-pregnant, female rabbit we got the following results:

Aorta.....	0.06 mg. $\text{NH}_3\text{-N}$ per 100 cc.
Renal vein.....	0.07 " " " 100 "

From the goat we obtained the following results:

Left kidney (with asepsis):

Arterial (carotid).....0.05 mg.  $\text{NH}_3\text{-N}$  per 100 cc.

Renal vein.....0.05 " " " 100 "

TABLE XI.  
*Urine of Goat 1.*

Volume.	Reaction (litmus).	Ammonia nitrogen.	Ammonia nitrogen, 24 hr. amounts.
cc.		mg.	mg.
80	Acid.		
40	"	72.7	
15	"	15.0	134.7
95	"	47.0	
35	Neutral.	1.61	
270	Alkaline.	6.4	
80	"		
40	"		
25	Neutral.	1.3	10.18
37	Alkaline.		
168	"	2.48	
135	"	1.51	
148	"	1.89	
75	"	0.38	
70	Acid.	1.90	11.22
250	Alkaline.	4.20	
75	"		
60	Neutral.	0.98	
60	"	0.36	
210	"		
250	"	3.45	
890	Alkaline.	8.01	
56	"	0.43	12 80
120	"	0.49	
80	"	0.42	
125	Neutral.	1.00	
85	"	1.17	
127	Acid.	72.97	72.97
137	"	86.7	86.7
122	"	19.0	
138	"	89.6	108.6
202	"	136.9	Incomplete.
50	"	13.1	"
240	Neutral.	33.6	"
47	"	4.3	Postmortem (bladder).

and 3 days later we found:

Renal artery.....	0.046 mg. $\text{NH}_3\text{-N}$ per 100 cc.
“ vein.....	0.068 “ “ “ 100 “

At the same time we obtained from the goat:

Femoral artery.....	0.048 mg. $\text{NH}_3\text{-N}$ per 100 cc.
“ vein.....	0.049 “ “ “ 100 “
Jugular “ .....	0.053 “ “ “ 100 “

While this work was in progress, Loeb, Atchley, and E. M. Benedict (6) published an article showing the arterial and venous blood of the kidney of the rabbit to have substantially the same concentrations of ammonia.

*Evidence That Increases in Blood Ammonia Values Do Occur in Nephritis.*

It is not surprising that the literature is in utter confusion with respect to blood ammonia values in pathological states. It is doubtful if the method should ever be applied in the average clinical investigation. One finds such absolutely unreasonable values reported for normal blood (Hara (22)) that the variations in pathological conditions are not convincing.

Gherardini (23) states that the ammonia content of blood is not increased in many forms of nephritis. Rabinowitch, in investigating a considerable number of cases of nephritis and diabetes in a study of the origin of urinary ammonia (8) found no increases in blood ammonia in any of his cases. Others have reported increases of a bizarre nature.

Our animal experiments, after months and years of painstaking repetition, carried the conviction that blood ammonia increases in human nephritis might well be missed through the failure to consider the rôle of vomiting in ammonia elimination in the way that it had been ignored experimentally. Through the courtesy of Dr. E. P. Richardson and the residents and house officers of the Massachusetts General Hospital this point was studied in detail.

The method of making the clinical observations is of the greatest importance and justifies detailed description. When a blood ammonia determination was to be made on a patient, a special nurse was kept with the patient to record the time of vomiting, if any, and the separate collections of the vomited material was thus insured. The author prepared fresh re-

agents and ran a control on the reagents immediately before the blood was drawn from the patient. It was so arranged that the author's blood was drawn as a normal control a few minutes before he took the blood from the patient and in all cases his own ammonia-free potassium oxalate was used

TABLE XII.  
*Vomitus and Blood Ammonia Values in Nephritis.*

Sub- ject.	Vomitus.			Blood.				Remarks.
	Vol- ume.	Reac- tion.	NH <sub>3</sub> -N	Non- protein nitro- gen.	Uric acid.	Creat- inine.	NH <sub>3</sub> -N	
	cc.		mg.	mg.	mg.	mg.	mg.	
A	126	Acid.	80					Dec. 18
	86		35	158	16.6		0.29	" 19
				227	26.6			" 23
B						Died.		" 28
				208	4.3	14.5	0.10	Just after vomiting, Jan. 26.
	120	"	70					Jan. 29, taken home to die.
C	470	"	145	149	8.0	4.2	0.16	Oct. 21, 2-7 p.m., overnight up to
	720		200					7 p.m., Oct. 22.
	1370	"						Oct. 24, 18 hrs. end- ing 7 a.m.
	520		226					6 hrs. ending 1 p.m.
	850	"	238					5 " " 6 p.m.
	240	"	125					
				143	8.8	4.2	0.17	Oct. 25
				Period of convalescence.				
				105	4.6	3.3	0.10	Nov. 10
				No vomiting—improvement.				
				6 mos. interval—then readmission.				
	67	"	97					Apr. 6
				Fatal termination.				

*Subject A.*—Mrs. B. K. B. (252772), age 33, chronic nephritis, edema. Food had been refused for over a week preceding the data.

*Subject B.*—Mr. C. H. (267535), age 31, chronic nephritis. Jan. 29, patient had been vomiting repeatedly. Sample of 120 cc. represented but a short period of accumulation.

*Subject C.*—Mr. G. DeM. (251074). This was a very interesting case and one that afforded a long period of careful study. He was intelligent and cooperative. One tuberculous kidney had been removed at least a year previous—the remaining kidney was tuberculous (autopsy) and its failure to function precipitated uremia on various occasions. There is only space for a small amount of the data that were obtained on this case.

to prevent clotting, and the blood was taken in his own syringes. Alongside of the normal control, the patient's blood was introduced into the aeration apparatus, previously arranged so as to be ready for instant aeration. It is almost impossible to overemphasize the need for absolutely convincing control work in these determinations. A single determination invariably used up half a day's time.

A study of cases in the hospital other than nephritics showed that vomitus obtained spontaneously as well as material from gastric lavage contained but negligible quantities of ammonia. (It is specifically emphasized that very minute amounts of ammonia nitrogen in vomitus of controls have no significance, for our vomiting nephritics put out as much as several hundred mg. in a few hours.) This was in accord with the large number of observations we had made on experimental animals. Our results with vomiting nephritics gave a very clear-cut picture (Table XII).

Thus, our human cases show a picture identical with that found in experimental animals—increases in blood ammonia values when taken before vomiting; the vomiting of quantities of ammonia that are comparable to what might be eliminated in the urine for the same period; and a return to normal ranges of values with convalescence. (It did not seem to be in the interest of the patient to stop alkali administrations, and for that reason ammonia determinations on the urine would have been valueless, quite aside from the difficulties encountered in incontinence.)

It seems impossible to escape the conviction that our animal experimentation ran a course of events very similar to what we saw in the carefully studied patient.

It must be admitted that the problem of ammonia formation admits of clearer, more direct, and hence more convincing proof in experiments dealing with concentrations found in circulating blood than in any other type of data. Clinical material by virtue of its very abundance lends itself to much speculation. It can be little more than that as long as there are the vast array of factors that may and must come into play between ammonia formation and its final excretion, to say nothing of the difficulties that are inherent in the hospital management of routine determinations that are done on a large scale. Fiske (24) has reviewed a number of factors involved in interpreting the excretion of ammonia, some of which cannot possibly be controlled in hospitals, if indeed in any but the most heroic humans.

## DISCUSSION.

The formation of ammonia for the neutralization of acids is not the function of any particular tissue—either in the normal or the nephritic dog. Our findings convince us that just as acid production goes on throughout the body, so does ammonia formation accompany it.

The important conclusion of Nash and Benedict is “that the kidneys themselves must produce the urinary ammonia.” They reached this conclusion as the result of finding more ammonia in the renal vein than in the systemic circulation, and found conclusive evidence for this view in their experiments with phlorhizin-poisoned dogs, and dogs that survived double nephrectomy, and in some cases double ureter ligation, for from 44 to 71 hours.

While, at first glance, this might seem to furnish the necessary proof for their contention, we believe that their results, as well as ours, may be interpreted in such a way as to throw considerable doubt upon the conclusions they have drawn from them. Both their results and ours agree in that the renal vein contains more ammonia than the renal artery, and this certainly means that the kidney forms some ammonia. But, a study of their experiments with double nephrectomy fails to disclose in them conclusive proof that this ammonia-forming function is necessarily limited to the kidneys.<sup>4</sup>

Whoever has worked with doubly nephrectomized dogs must have been impressed with the fact that they continually drink and vomit, and, in experiments lasting from 2 to 3 days it is entirely possible, and indeed now proven, that ammonia excretion by way of the vomitus keeps pace with ammonia formation and may result in an equilibrium whereby the level of ammonia in the blood remains at its normal figure. That this interpretation of the results is the correct one is further borne out by the fact that in acute experiments where vomiting does not occur and ammonia elimination is really excluded, double nephrectomy is accompanied by the significant increases in blood ammonia seen in Table VIII, where the preliminary value of 0.13 mg. increased

<sup>4</sup> At the present time experiments are under way which have been designed to yield information as to the relative quantitative importance of the kidney and other ammonia-forming organs.



to 0.23 mg. when vomiting stopped. In Table IX the preliminary value of 0.09 mg. was nearly trebled, reaching the value of 0.25 mg. 6 hours after the last previous vomiting. That vomiting is able to keep the level of ammonia in the blood at the normal figure is shown in Table X where the twice normal value of 0.19 mg. was reduced by vomiting to 0.08 mg.

Our results with phlorhizin-poisoned dogs are in agreement with those obtained by Nash and Benedict, but we see no argument here for localizing ammonia formation in the kidney. The plasma  $\text{CO}_2$  values of 38.1 to 54.1 reported by them, in spite of high urinary ammonia values, cannot be interpreted as indicating a severe phlorhizin acidosis, and the normal values obtained by them and us are quite to be expected in the absence of a marked acidosis.

The extra-renal formation of ammonia is not difficult to demonstrate. Dog 27, after ligation of both kidneys, increased the arterial value from 0.21 to 0.35 mg. after 1 hour of ligation, while Dog 28 showed the same proportional increase from 0.09 mg. to 0.14 mg. after 90 minutes of ligation. It is interesting in this experiment to note that the only time that the spleen appears to be as important a contributor of ammonia as the other organs in question is after the ligation of the left and right renals and the pancreaticoduodenal vein.

Surveying the results obtained with blood from various types of tissue we notice that the net effect of having blood enter and leave muscular tissue is for it to decrease, even if but slightly, in ammonia content. These effects are more pronounced when there is arterial accumulation of ammonia. Glandular tissue produces more ammonia than other tissues. The spleen functions but weakly as compared with the pancreas and kidney, though there is the suggestion that it may assist in ammonia formation when the stress of circumstances warrants it.

The carotid and jugular values, measuring the net effect of having blood enter and leave both muscular and glandular tissue, reveals an even balance until acidosis calls forth more ammonia from venous blood.

Nash and Benedict say that, "If ammonia were formed in the organism in appreciable amounts elsewhere than in the kidney, we should expect the injection of acid into the circulation to be

followed by a definite increase in the ammonia of the general systemic blood." We have shown that a physiological influx of acids does increase the concentration of ammonia in the blood when means are devised for showing it. Nash and Benedict were unable to demonstrate this, and the violent dyspnea reported in their animals when acids were injected reminds us that such violent breathing causes such a great removal of acid from the blood that it is possible by this means to change the reaction of the urine from acid to alkaline, and this condition would not be reflected in their measurement of the  $\text{CO}_2$ -combining power of the blood which they used as a measure of their acidosis.

The experiments with ammonium salts indeed raise the question as to whether further increases in acids produce further increases in the level of ammonia in the blood after it reaches a certain value, somewhere below the concentration at which its toxic effect becomes pronounced. Certainly acidosis is more readily tolerated than are excessive concentrations of ammonia. Were there no chemical reason for supposing that ammonia accumulation in the blood must find a *low* maximum limit, the pharmacological action of the ammonium ion is such as to prohibit its undue accumulation in the blood. Ammonium salts are well known to be toxic. However excessive the formation of acids or however urgent the call for alkalis to neutralize them, the competition of bases for the acid radicles will be determined by the relative concentrations and strengths of the bases present. Any protective device that operates to keep down excessive accumulations of the toxic ammonium ions will, in just that measure, call more and more upon the fixed bases of the body—with a consequent diminution of the alkaline reserve. If ammonia formation, in itself, were an adequate means of neutralizing acids in severe acidosis there would be no reduction of the alkaline reserve of the blood and tissues.

Our experiments with dogs in the acidosis of an extreme nephritis give a more rational picture of the changes that occur in this condition.

Relative to ammonia formation it may be maintained that the ammonia content of the renal vein merely represents the escape of ammonia from the kidney and that this portion may be only an insignificant part of the whole amount formed, the major portion

of it going directly into the urine. This assumption, however, would be difficult to prove. In fact our evidence of accumulation of ammonia in the blood seems to admit of the interpretation that ammonia, like other nitrogenous constituents of the blood, finds its way into the urine in a manner similar to that of other metabolic wastes. Dog 30 furnishes evidence in support of this idea. This dog, with an acute renal insufficiency, eliminated only 62 cc. of urine in 3 days, but kidney function, as shown by the minimal urine output and the high non-protein nitrogen values of 250, 357, 391, and 655 mg., was so deficient that in 3 days but 8.1 mg. of ammonia nitrogen were eliminated in the urine as compared with 240.1 mg. eliminated in the vomitus.

By calculations based on the *probable* rate of blood flow through the kidneys, Nash and Benedict have attempted to show that pathological increases in urinary ammonia are not explainable in the light of such data. Since 1921 the methods for determining the rate of blood flow have shown (25) that under various conditions the rate of blood flow is increased many more times than is necessary to account for the elimination of ammonia from arterial blood values. Even if the subject of the rate of blood flow differs in no respect from what it was in 1921, there is no doubt but that the clear-cut results obtainable from blood and vomitus analyses are more convincing in nature than such uncertain calculations from data that were not intended to be capable of serving as the basis for the drawing of such fine distinctions.

In comparing the dog and the goat, the most striking difference is in the absolute values for blood ammonia. The dog with a strongly acid urine carries just double the concentration of ammonia in his systemic circulation that we find for the goat. The goat normally secretes an alkaline urine with but traces of ammonia.

With a blood ammonia method that is continually controlled, and where blanks continually show less than 0.005 mg. of ammonia nitrogen per 100 cc., we believe that the difference between 0.05 mg. in the goat and 0.10 mg. in the dog is a real one. Our results suggest that under conditions of normal kidney function, ammonia excretion from the blood is accomplished with an extraordinary degree of completeness and facility, and that the ammonia in systemic blood represents what is formed throughout the body for

acid neutralization, and is there on its way to the kidney for excretion. Benedict first called attention to the urinary elimination of uric acid by the Dalmatian dog. This excretion of large quantities of uric acid from but traces in the blood has a striking resemblance to the conditions obtaining with ammonia.

#### SUMMARY.

1. Low absolute values for blood ammonia confirm Folin and Denis and Nash and Benedict.

2. Nash and Benedict's values for the ammonia content of renal venous blood are confirmed.

3. The author disagrees with Nash and Benedict's interpretation of the kidney as the "center of ammonia production in the body."

4. Evidence is reported to show that ammonia formation is a generalized tissue phenomenon.

5. Attention is directed to the important rôle of vomiting in the elimination of ammonia.

6. The parallelism of human pathology with the results of animal experimentation has been demonstrated.

7. Conclusions are drawn relative to the mode of excreting ammonia.

8. There is suggested a shift of emphasis from the ammonia content of venous blood to that of *arterial*.

9. It is shown that blood ammonia values do increase in nephritis.

This investigation was carried out under the closest cooperation with Professor Folin. His share in whatever may be of value is very large. The author claims responsibility for whatever mistakes there may be. I want to thank Professor Folin for his generous help and criticism and advice throughout the investigation.

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# **TOTAL ACID-BASE EQUILIBRIUM OF PLASMA IN HEALTH AND DISEASE.**

## **I. THE CONCENTRATION OF ACIDS AND BASES IN NORMAL PLASMA.**

**By JOHN P. PETERS, HAROLD A. BULGER, ANNA J. EISENMAN,  
AND CARTER LEE.**

*(From the Department of Internal Medicine of Yale University and the Medical  
Service of the New Haven Hospital, New Haven.)*

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### **INTRODUCTION.**

An extensive study of the electrolytes of the blood has been carried out on a large number of cases in the course of the last 3 years. The primary object of the work was to gain an insight into the mechanism of the changes in blood and tissue hydration in nephritis and cardiovascular diseases. The study has not been confined to an investigation of these conditions alone, but analogous material of all kinds has been investigated as occasion offered in the wards of a general hospital, in order to obtain adequate control material and to see whether changes encountered in a given disease were distinctive of that disease or whether they were associated with one or another symptom complex that the patient happened to present.

In all cases chloride and bicarbonate and in most instances the proteins of the plasma have been determined. These are the principal acids that occur in the plasma of normal blood. In a considerable number of experiments the plasma was also analyzed for inorganic phosphate. In the more recent studies the total base of the plasma has been determined by a modification of Fiske's method for the estimation of total base in urine. The complete procedure gives a picture of the total acid-base equilibrium of the blood. Additional studies have been added as they seemed indicated in individual cases.

The present section deals with studies of healthy adults under normal conditions and describes in detail the experimental methods employed.

### *Experimental Methods Employed.*

Blood was withdrawn from the artery or vein of the patient, usually before breakfast in the morning. In cases of emergency it was sometimes impossible to consider time or meals. These instances are noted in the case reports. Two techniques were followed in the treatment of the blood. In some cases the blood was withdrawn without contact with air and immediately placed over mercury, by the procedure described by Austin, Cullen, Hastings, McLean, Peters, and Van Slyke (2). In other instances the blood was withdrawn without precautions against air contact and then saturated with a known tension of  $\text{CO}_2$  in air at  $38^\circ\text{C}$ . by a technique previously described by the authors (18). It was then transferred to the regular mercury sampling tubes from which samples were withdrawn for analysis as required. The treatment to which the blood was subjected is indicated in the tables. *A* and *V* represent arterial and venous blood respectively. *Cont.* indicates that the blood was analyzed as drawn, while *cap.* specimens were saturated with 40 mm. of  $\text{CO}_2$  at  $38^\circ\text{C}$ . Usually the tension chosen was 40 mm. of  $\text{CO}_2$ . Sometimes the blood was saturated at both 30 and 60 mm. In this case 40 mm. values were estimated by the straight line logarithmic formula of Peters, Eisenman, and Bulger (19).

In most of the experiments the original receiving tube was coated with enough recrystallized, *neutral*, potassium oxalate to make a final concentration of 0.2 per cent in the blood. In some of the later experiments defibrination was effected without contact with air by a method devised by one of the authors and described elsewhere (11). This consists simply of almost entirely filling the regular mercury sampling tube with fresh blood. The tube is then repeatedly and forcibly inverted so that the small amount of mercury remaining in it is driven alternately from one end of the tube to the other through the column of blood. The mercury itself acts as the defibrinator and becomes intimately adherent to the clot, in fine globules. The defibrinated blood can be expelled from the tube in the usual manner while the clot stays behind with the mercury.

It is well recognized that the addition of oxalate to blood causes the cells to shrink appreciably. This affects not only the hematocrit value, but to a lesser extent, also, the distribution of all the electrolytes between the cells and the plasma. The average reduction of cell volume caused by the addition to the blood of 0.2 per cent oxalate has been estimated by one of the authors (11) and amounts to about 2 volumes per cent with considerable variations about this mean. The change in  $\text{CO}_2$  and chlorides is quite capricious, but usually much less than the change of cell volume. In the experiments in which serum was used and which are marked *s* in the protein column (No. 4), cell volume values are relatively higher and are comparable to the oxalated blood values.

The oxygen capacity was determined in the Van Slyke pipette by a method devised by Lundsgaard and Neill<sup>1</sup> which permits the saturation of the blood in the pipette itself. The method gives values that agree with those obtained by the procedure of Van Slyke and Stadie (25). Frequently, when the amount of blood available was small the packed cells from which the plasma had been removed were utilized for oxygen capacity determinations. In this case the blood was centrifuged in tubes with contracted necks. Before the plasma was removed the upper level of the liquid was marked by a piece of gummed paper. After the plasma had been removed the volume of the solution was made up to the original level with normal saline solution, the packed cells were stirred up until an even emulsion was made, and samples of this emulsion were taken for analysis. Although this method does not always give values identical with those derived from the original blood, the checks are satisfactory for the purpose for which they were intended in this work.

Cell volumes were determined in duplicate with a Daland hematocrit of the type manufactured by the International Instrument Company for their centrifuge. This was rotated at about 2000 R.P.M. until the cells were homogeneous and translucent and showed no tendency to further reduction in volume. Duplicate determinations usually differ by considerably less than 1 volume per cent and it is probably justifiable to assume that for comparative purposes this represents the upper limit of error in the actual determination.

Carbon dioxide was determined by the method of Van Slyke and Stadie (25) in a carefully calibrated, water-jacketed, Van Slyke pipette, graduated in 0.01 cc.

For the estimation of chlorides the method of Austin and Van Slyke (3) with certain modifications was employed in the earlier part of the work. These modifications, devised by Austin,<sup>2</sup> consisted essentially in making the original dilutions in calibrated centrifuge flasks and removing the precipitates by centrifugation. A more dilute iodide solution was employed for the titration and only 1 cc. of plasma or blood was taken for each analysis. In the latter part of the work, beginning September 1, 1923, the newer procedure of Van Slyke (24) was substituted for that of Austin and Van Slyke. Van Slyke's last method gives values that are more accurate and a trifle lower than those obtained by the earlier picric acid precipitation procedure, but the difference is not highly significant for the present purposes.

Phosphates were determined at first by Briggs' (7) modification of Lell and Doisy's method. Later the new procedure of Benedict and Theis (6) was employed.

For pH the colorimetric procedure of Cullen (9) was used. In a large part of the early work the indicator solution was too acid and the pH values

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<sup>1</sup> Personal communication.

<sup>2</sup> Personal communication.



obtained were accordingly 0.0 to 0.2 higher than they should have been, the error increasing with the pH. These values are indicated in the tables by the mark.\* The error, although it destroys the absolute value of the pH figures, does not seriously alter the results of the acid-base calculations. The figures have been retained because they indicate the nature and direction of changes in hydrogen ion concentration in the different experiments.

For total base a method devised by Cullen and Robinson<sup>3</sup> was employed. This is an adaptation and modification of Fiske's (12) method for the determination of total base in urine. In this procedure the phosphorus is supposedly completely converted to the meta-phosphate form and does not react with benzidine, but combines with one equivalent of monovalent base. Separate determination of inorganic phosphate is, therefore, required. In our experience the presence of excessive amounts of phosphate diminishes the accuracy of the method to a certain extent which cannot be accurately predicted. The error is not great enough, however, to invalidate any of the conclusions drawn in the following papers.

Calcium was determined by the method of Kramer and Tisdall (23).

Whole blood only was analyzed for non-protein nitrogen and blood sugar. A Folin and Wu tungstic acid filtrate or a filtrate obtained by trichloroacetic acid precipitation of the proteins was subjected to micro digestion by the usual procedure. The ammonia was distilled into 0.02 N acid and estimated by titration with 0.02 N alkali.

For the determination of the proteins 0.5 cc. of plasma or serum was subjected to the macro Kjeldahl procedure. From the total nitrogen the non-protein nitrogen of the blood was subtracted. The remainder was multiplied by the usual factor, 6.25.

For blood sugar the method of Folin and Wu (13) was employed in all but a few of the early determinations, when Benedict's (4) modification of the Lewis and Benedict method was used.

Uric acid was determined by the direct method of Benedict (5).

### *Calculations Employed.*

With few exceptions only those experiments have been presented in which sufficient data were obtained to permit a reasonably accurate estimate of either the total inorganic acids or the total base or both. Often, for one reason or another it was impossible to obtain sufficient plasma or serum to carry out all the procedures involved in a total acid analysis. In these cases certain factors have been employed to fill in the deficiencies.

In the estimation of *proteins from total serum nitrogen* the non-protein nitrogen was determined whenever there was the least reason to suspect that it might be high. When this analysis was omitted a standard correction of 0.19 per cent (corresponding to 30 mg. per cent of non-protein nitrogen) was subtracted from the protein equivalent of the total nitrogen of the plasma.

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\* Personal communication.

If the serum or plasma was not analyzed for total nitrogen, a protein value of 7.00 per cent was assumed. The value for protein was converted into millimols of base combined with protein by the formula of Van Slyke, Wu, and McLean (26).

$$B_p = 0.68 P_p (\text{pH} - 4.80) \quad (1)$$

in which  $B_p$  and  $P_p$  represent respectively millimols (mm) of base combined with protein and per cent of protein in the serum or plasma.

The failure to determine non-protein nitrogen in some experiments and the use of non-protein nitrogen values derived from whole blood in the others is not entirely justifiable, but was necessary for reasons of economy. Presumably the non-protein nitrogen of the plasma would be somewhat lower. The assumption of a pH value of 7.35 in those experiments in which no colorimetric or gasometric determination was made introduces a variable error which probably seldom exceeds 1 millimol. This error is further reduced by the fact that the plasma proteins are frequently low in conditions in which the pH is abnormal. The assumption of an average value of 7.0 per cent for proteins when the latter are not determined is open often to still more serious criticism. However, protein analysis has seldom been omitted when there was any reason to expect a serious protein abnormality. Of course there is a difference between serum and plasma proteins and it is not quite proper to employ the two interchangeably as we have done. Under ordinary circumstances we have found that the effect of oxalate in causing the cells to shrink and thus diluting the plasma practically compensates for the loss of protein resulting from defibrination, so that plasma and serum proteins are for all practical purposes equal. In the presence of infection, especially pneumonia, and certain other diseases, however, the fibrin may be increased to such an extent that the serum proteins may be considerably less than those of plasma. In the case of the observations on pneumonia in this series, where serum has been used, the proteins of the plasma have also been determined and the latter value has been used for the calculation of the acid value of protein.

All these sources of error are really quite insignificant in comparison with the uncertainty of the equation which has been employed for the determination of the base-combining power of the protein. This equation was derived by Van Slyke, Wu, and McLean (26) from some experiments on the blood of a single Manchurian pony. Its applicability to normal human blood serum is highly questionable. Its use for blood obtained from patients with diseases which alter the relative proportions of albumin, globulin, and fibrin probably entails further error. Obviously, however, no estimation of the total acid of plasma that fails to take into account the acid value of the proteins is satisfactory. The equation is at least qualitatively an expression of the truth and offers an approximation that is probably satisfactory for comparative purposes, at least.

In the *cont.* experiments base present as bicarbonate was calculated by the following equation:

$$\text{CO}_2 - \left[ \frac{14.04 \text{ CO}_2}{2.24 \times \text{antilog}(\text{pH} - 6.1)} \times 0.713 \right] \quad B \text{ as } \text{BHCO}_3 \quad (2)$$

where  $\text{CO}_2$  = volumes per cent of total  $\text{CO}_2$ ,  $\frac{14.04 \text{ CO}_2}{\text{antilog}(\text{pH} - 6.1)}$  = the  $\text{CO}_2$  tension in mm. of mercury, 0.713 = the coefficient of solubility of  $\text{CO}_2$  in serum at  $38^\circ\text{C}$ ., 2.24 = the gas constant, and  $B$  = the millimols (mm) of base combined with  $\text{CO}_2$  as bicarbonate.

In *cap.* experiments  $B$  as  $\text{BHCO}_3$  is easily calculated by the equation

$$B = \frac{\text{CO}_2 - 2.85}{2.24} \quad (3)$$

where 2.85 volumes per cent = the amount of  $\text{CO}_2$  dissolved in serum at 40 mm. of  $\text{CO}_2$  tension and  $38^\circ\text{C}$ .

The *phosphorus* in mg. per 100 cc. was reduced to milli-equivalents per liter by the factor  $\frac{18}{31.04}$ , accepting L. J. Henderson's (15) estimate of the proportions of primary and secondary phosphate in the blood. When the phosphate was not determined a value of 3.5 mg. per cent was assumed.

In the *cap.* experiments pH was calculated by means of the Henderson-Hasselbalch equation,  $\text{pH} = \text{pK}_1 + \frac{\text{BHCO}_3}{\text{H}_2\text{CO}_3}$ , accepting as a value for  $\text{pK}_1$ , 6.10. If pH was not determined a value of 7.35 was assumed for purposes of calculation.

The *total acid* is calculated as the sum

$$TA = B_P + B_{\text{CO}_2} + B_{\text{Cl}} + B_{\text{PO}_4}$$

where  $B_P$ ,  $B_{\text{CO}_2}$ ,  $B_{\text{Cl}}$ , and  $B_{\text{PO}_4}$  represent millimols of base combined with protein,  $\text{CO}_2$ , Cl, and phosphate respectively and  $TA$ , therefore, the total base-combining power of the inorganic acids and the protein of the serum or plasma.

The difference between this and the total base in the preceding column, which in the tables has been termed "organic acid", represents the amount of base present as sulfates and salts of organic acids.

Failure to determine inorganic sulfur makes the sum total of the acids incomplete and to that extent any conclusions concerning the acid-base balance must be considered as tentative. Under ordinary circumstances the concentration of inorganic sulfur in the blood is negligible. Denis (10) has reported as much as 20 mg. per cent in cases of nephritis with non-protein nitrogen retention, an amount that would affect appreciably the acid values in these studies. If it is assumed that each atom of sulfur combines with two equivalents of monovalent base 20 mg. of inorganic sulfur represent 12.5 millimols of combined base.

*Concentration of Acids and Bases in Normal Human Plasma.*

In Table I appear the results of studies made on ten healthy adults, for the most part members of the staff of the medical department of the university, or medical students. Protocols of these subjects are given below. All determinations except those of CHP, the first eight on JP, the second of HAB and that of ABD were made before breakfast, and for all the latest procedures were employed. These represent better than do the data in the subsequent tables the variations that one may expect to find in normal resting individuals when blood is withdrawn without stasis and analyzed by the most accurate clinical procedures.

Objection may be made to the inclusion of All as a normal because he presented an albuminuria and low serum proteins at the time of the first study. The albuminuria was minimal and transitory, probably a sequela of a slight respiratory infection, and would have escaped the notice of anyone but an introspective medical student. It is questionable whether it had any connection with the low serum protein. Tha showed proteins quite as low in the absence of albuminuria or any other sign of illness. Linder, Lundsgaard, and Van Slyke (17) observed a temporary protein reduction of the same degree in a perfectly healthy individual. On a subsequent occasion All's proteins had risen to the usual level. CFM should not, perhaps, be included in this table. Apparently a mild anemia and hypothyroidism controlled by thyroid medication do not affect the acid-base equilibrium appreciably because all analyses fall well within the normal limits. If this observation and the one made on JP on February 23, 1925, were omitted the conclusions would be essentially unaltered.

With the exception of the first study of All the total base is remarkably constant, remaining always within the narrow limits, 147 to 161 millimols. This one exception is almost certainly due to an analytical error because total base is smaller than "total" acid in this experiment, an impossible condition in view of the fact that the serum is alkaline. These values are quite similar to those reported by other observers who have determined the different basic elements separately. Kramer and Tisdall (16)

**TABLE I.**  
*Concentration of Acids and Base in the Blood of Normal Adults.*

Subject.	Date.	Oxygen capacity.	Cell volume.	Ratio of Columns 1:2.	Plasma.										Blood non-protein nitrogen.	Treatment of blood.	Remarks.
					Protein.	CO <sub>2</sub>	Cl	Inorganic P.	Total acid, Columns 4+5+6+7.	Total base.	Organic acid, Columns 9-8.	pH					
		vols. per cent	vols. per cent		per cent	vols. per cent	mg. per 100 cc.	mg. per 100 cc.	mm	mm	mm	mm		mg. per 100 cc.			
Ehr	1945 Apr. 7	17.4	43.9	39.6	7.11 s	69.1	362	3.9	145.7	154.7				34	V cont.	Before breakfast.	
Hal	" 8	18.4			6.59 s	67.5	370	4.3	145.7	154.0				35	"	"	
HAB	" 21				7.11 s	59.1	362	4.0	141.4	147.2				31	"	"	
	June 26	17.4	43.9	39.6	7.36 s	65.9	348	3.4	139.7	149.1			7.26		"	1 hr. after breakfast.	
Tha	Apr. 27	19.7	47.3	41.7	5.76 s	71.4	369	4.3	147.4	161.3				31	"	Before breakfast.	
AJE	" 10	17.3	41.3	42.0	7.72 s	59.0	362	3.1	141.1	152.9				31	"	"	
CHP	" 24	16.3	41.2	39.6	6.62 s	63.7	369	3.8	144.4	156.2				34	"	1 hr. after breakfast.	
All	Feb. 19	18.6	46.0	40.5	5.82 s	73.1	370	3.9	147.5 (135.1)			(-12.4)		29	"	Before breakfast.	
	May 7	18.8	44.8	42.0	6.90 s	68.5	372	2.9	147.3	159.0				37	"	"	
	1944 Mar. 11	18.6	42.7	43.6	6.71	60.8	370		143.5				7.34		"	About 1 hr. after break-fast.	
JP	" 27	18.2	42.6	42.8	6.43	51.5	380		141.8				7.33		" cap.	"	
	" 18	18.4	41.4	44.5	6.62	56.3							7.40		"	"	



found 156 to 159 mm in a series of normal adults; Briggs (8) obtained 136 to 163 mm in a study of a large number of hospital patients with various pathologic conditions other than nephritis; Salvesen and Linder (22) found 151 to 162 mm in seven normal individuals.

The "total" acid varies even less than the total base in this series, being always within the limits 138 to 148, or about 7 per cent. Apparently there is no consistent relation between the levels of base and acid. The consequence is that "undetermined" acid is more variable relatively and absolutely than any other constituent estimated or the sum of these constituents. In these experiments the undetermined acid ranges from 5.8 to 19.8 mm.

TABLE II.

*Variations in the Concentration of Acids and Base in the Blood of Normal Individuals.*

Plasma acids.	Maxi- mum.	Mini- mum.	Aver- age.	Average variation.		Total variation.	
	mm	mm	mm	mm	per cent	mm	per cent
Protein.....	13.4	10.0	11.4	±0.6	±5.5	3.4	34
Bicarbonate.....	30.9	21.8	25.8	±2.6	±11.9	9.1	39
Chloride.....	110.1	100.0	103.5	±2.8	±2.8	10.0	10
Phosphate.....	2.7	1.7	2.3	±0.2	±13.7	1.0	59
"Total" acid.....	147.5	137.8	143.3	±2.7	±1.9	9.7	7
Total base.....	161.3	147.2	155.7	±2.9	±1.9	14.1	10
Undetermined acid.....	19.8	5.8	12.1	±3.2	±55.6	14.0	241

Of the other acid constituents the phosphates are relatively most variable, but appear in such low concentration as to have little influence on the total acid. Bicarbonate is, as one might expect, responsible for the major part of the changes in "total" acid, illustrating what Gamble, Ross, and Tisdall (14) have called the "mendicant position of bicarbonate." The protein shows a relatively large fluctuation, but its absolute effect on the acid sum is not as great as that of the chlorides. The sum of the variations of the individual acids is far greater than that of the "total" acid. In the experiments on the group and also in the series of studies on JP there is a tendency for bicarbonate, chloride, and protein variations to compensate for one another. This becomes more apparent in comparisons of individual experi-

ments. It is best illustrated, perhaps in the two determinations on All. In these protein, bicarbonate, chloride, and phosphate all change, while the sum of the acids remains constant.

The total limits of variation of the different constituents are shown in Table II.

In Table III is another series of determinations from a group of normals and patients with pathologic conditions which are not of such a nature that they would be apt to alter the electrolyte equilibrium. For the first sixteen observations the chloride method of Austin and Van Slyke was employed. This explains the higher general level of chlorides observed in the series and largely accounts for the higher concentration of "total" acid. This group of determinations is included as a basis of comparison for the studies on pathologic cases in which the same methods were employed. The proteins are also somewhat higher than those in the preceding table and to many may seem more like the values with which they are familiar. The difference is probably referable to the technique employed in the collection of the blood. It was originally pointed out by Rowe (21) and has recently been confirmed by the authors (20) that the production of venous stasis leads to a local concentration of the blood, which exhibits itself in an augmentation of the plasma proteins. In the experiments in the first table no stasis was produced. The bloods of the earlier series, represented by the first sixteen studies in Table III, were intended largely for other purposes. For this reason and because, at the time, the full effect of stasis on the composition of the blood was not appreciated, a tourniquet was applied to the arm to facilitate the collection of blood. The influence of venous stasis on plasma protein concentration has been too little recognized. Atchley, Loeb, Benedict, and Palmer (1), for example, present normal values much more like those of Table II than those of Table I. They state that a tourniquet was applied to the arm to produce stasis, but the pressure was not maintained longer than 2 minutes. In our experience this is quite long enough to cause considerable concentration of the blood.

Samples 17 to 24 inclusive were studied by the more recent procedures and are presented largely for the total base determinations. The variation of total base is larger than that found





	1923	18.3	38.6	47.5		68.3	356		142.3	7.44*		A cont. V	Before breakfast.
18267	Apr. 11	19.2	39.2	49.1	6.30	69.9	355		142.9	7.42*		"	"
18373	" 25	19.6	40.7	48.2		60.9	378		145.2	7.41	30	"	"
	1924.												
26871	Jan. 10	17.3	38.9	44.5	6.73	49.5	362		147.6	7.31	31	" cap.	"
29822	Apr. 9								157.0			" cont.	"
29882	" 16								160.0			"	"
26839	May 22							4.9	149.4		31	"	"
	" 26							3.9	164.8			"	"
271	" 25							4.5	159.4			"	"
Nor								4.8	167.0			"	"

s in the protein column indicates that serum and not plasma was analyzed.

\* pH values followed by \* are 0.0 to 0.2 too high because the indicator solution used for these determinations was too acid.  
Cf. discussion of Methods.

in the normal individuals previously discussed. This greater degree of variability appears throughout this table. It is undoubtedly an expression of the fact that the subjects under investigation were more variable because of the pathologic conditions from which they suffered. The divergence from normality is so slight that it is impossible to connect the disturbance of equilibrium with any special phenomena of disease. It indicates, however, that the normal limits of variation which have been defined above must be extended somewhat before any attempt is made to analyze the changes that result from disease.

If we assume as the limits for total base 147 to 167 millimols and for "total" acid 135 to 155 millimols we shall probably include most of the variations which result from minor pathologic conditions and for which no cause can be readily discovered.

#### SUMMARY AND CONCLUSIONS.

An attempt has been made to study the total electrolyte equilibrium of the plasma in health and disease by determining at the same time the total base, the inorganic acids, bicarbonate, chloride, and phosphate and the protein. The present paper deals with healthy individuals under normal conditions and describes in detail the methods employed.

The difference between total base and the sum of the base-combining powers of the acids enumerated gives a measure of the organic acid and sulfate. The latter is present in negligible amounts only, so the "undetermined" acid must be practically equivalent to organic acid.

Normal serum contains 147 to 161 millimols of monovalent base. 138 to 148 millimols of this base are combined with the four acids, protein, bicarbonate, chloride, and phosphate. These limits should probably be extended somewhat for hospital patients to 145 to 167 for total base and 135 to 155 for total acid.

The organic acid never exceeded 20 millimols in normal persons or patients with pathologic conditions in which there was no especial reason to expect a disturbance of electrolyte equilibrium.

There is a general tendency for protein, bicarbonate, and chloride to reciprocate in their changes and to aid one another in maintaining the total acid and total base at a constant level.

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#### PROTOCOLS.

*Ehr.*—Male, Assistant Resident Physician, in good health.

*Hal.*—Male, medical student, in good health.

*HAB.*—Male, age 32, instructor in medicine, in good health.

*Tha.*—Male, medical student, in good health.

*AJE.*—Female, research assistant in chemistry, in good health.

*CHP.*—Female, age 35, married, in good health.

*All.*—Male, medical student. At the time of the first examination he showed a faint trace of albumin in his urine, but no evidences of organic disease of any kind. At the time of the second examination the albuminuria had disappeared and he seemed entirely normal.

*ABD.*—Male, physician, age 36. At the time of the blood examination he was suffering from a mild lumbago, but was otherwise in good health.

*JP.*—Male, physician, age 34, and in good health at the time of all the examinations except that of February 23, 1925, when he was suffering from a mild nephritis following an influenzal infection. At the time of the venipuncture he was afebrile and had no symptoms except pain in the lumbar region. The urine contained moderate numbers of red blood cells. This blood specimen was withdrawn before breakfast in the morning. All the others from this subject in both Tables I and II were taken 60 to 90 minutes after a light breakfast.

*CFM.*—Female, chemical technician, with mild hypothyroidism, adequately controlled by thyroid medication.

*Case 12016.*—Italian, male, age 41, admitted to the hospital for attacks of abdominal distress and vomiting, with considerable emotional instability, but no objective evidences of organic visceral or nervous disease. His blood Wassermann was positive, but the cerebrospinal fluid was negative. At the time the blood was examined he was entirely free from symptoms. *Impression:* Psychoneurosis, with hysterical trend; syphilis on the basis of positive Wassermann only.

*Case 10875.*—Polish, female, age 24, admitted to the hospital for nausea, unassociated with vomiting or anorexia, and mild, atypical convulsive seizures. Examination revealed a stocking anesthesia of the legs, absence of deep tendon reflexes, and a positive Wassermann. At the time of the blood examination she was free from symptoms. *Impression:* Syphilis of the central nervous system.

*Case 4729.*—Male, medical student, admitted to the hospital for investigation of a glycosuria which had been discovered in the course of a routine examination and which proved to be transitory and benign in character.

*HKB.*—Male, age 25, medical intern, in good health.

*MD.*—Female, age 22, chemical technician with mild hyperthyroidism.

*Case 10859.*—American, male, age 17, admitted to the hospital during a hysterical convulsion. Examination revealed no evidences of organic disease and the blood Wassermann was negative. At the time of the blood examination he had been free from symptoms for more than 24 hours.

*Case 10919.*—Male, age 40, brother of No. 77914, admitted to the hospital with cerebrospinal syphilis, glycosuria of a benign type, and mild alcoholism. For 2 months he had suffered from tabetic gastric crises, which he had treated rather freely with whisky. At the time of the blood examination (before breakfast), he was free from all symptoms, his blood sugar was 132 mg. per cent, and his urine contained neither glucose nor acetone.

*Case 77914.*—Male, age 44, examined because of a glycosuria which appeared to be benign. The blood sugar at the time of the blood examination, before breakfast, was 141 mg. per cent.

*Sco.*—Female, age 35, social worker, with psychasthenia, but no evidences of organic disease.

*Case 15004.*—Polish, female, age 30, admitted for vague abdominal pains which together with indefinite nervous symptoms, had followed a difficult labor with prolonged postpartum hemorrhages 9 months earlier. For a few days before admission she had noted twitching of her fingers and hands, which assumed a position suggesting carpopedal spasm. At the time of the blood study she was free from symptoms and presented no signs of tetany. *Impression:* Psychoneurosis.

*Case 12267.*—American, male, age 33, admitted after an alcoholic debauch. He had a long standing cardiac valvular lesion, rheumatic in origin, affecting both mitral and aortic valves. His liver was slightly enlarged and he was mildly cyanotic, but presented no other evidences of cardiac

decompensation. At the time of the blood examination, when the blood was withdrawn synchronously from the radial artery of one arm and the vein of the other (without stasis) he had been at rest in bed for 40 hours and was free from symptoms.

*Case 18373.*—Italian, male, age 47, with cystic glioma of the right frontal lobe, but no evidences of other organic disease.

*Case 26871.*—American, female, age 49, with a glioma of the left parietal lobe of the cerebrum but no evidences of other organic disease.

*Case 29822.*—Colored, female, age 41, with syphilitic optic neuritis.

*Case 29882.*—American, girl, age 17, with hyperthyroidism. At the time of the examination her basal metabolism was 32 per cent above normal. *Impression:* Exophthalmic goiter.

*Case 6839.*—German, female, age 62, poorly nourished, with chronic glaucoma of 2 years standing. Although no evidence of other organic disease was discovered and the Wassermann test in both blood and spinal fluid was negative, she was somewhat stuporous and so strikingly undernourished that she was suspected of having some other unrecognized condition. The first blood examination was made the morning after she entered the hospital; the second, 4 days later, when her general condition was much improved. *Impression:* Glaucoma; chief disease not diagnosed.

*Case 271.*—Italian, female, age 47, admitted because of slight abdominal pain following cholecystectomy and appendectomy performed 6 years before. No signs of organic disease were discovered, but the patient had a distinct psychoneurosis. At the time the blood was examined she was entirely free from symptoms.

*Nor.*—Male, medical student, examined for a glycosuria which appeared to be of a benign character.

## TOTAL ACID-BASE EQUILIBRIUM OF PLASMA IN HEALTH AND DISEASE.

### II. THE EFFECT OF CO<sub>2</sub> TENSION ON THE CONCENTRATION OF THE ACIDS OF THE PLASMA OF OXYGENATED BLOOD.

BY ANNA J. EISENMAN, HAROLD A. BULGER, AND JOHN P. PETERS.

(From the Department of Internal Medicine of Yale University and the  
Medical Service of the New Haven Hospital, New Haven.)

(Received for publication, August 3, 1925.)

In the first paper of this series (2) a method for the determination of the total acid-base equilibrium of the serum was described and the variations in the different acids and the base of the blood serum of normal, resting adults were defined. This procedure in its complete form consists of the simultaneous determination of the total base, carbon dioxide, chloride, inorganic phosphate, and protein. In the study of pathologic cases sometimes the blood has been examined as drawn with precautions to prevent contact with air and consequent alterations of gas tension, in other instances it has been analyzed after saturation with 40 mm. of CO<sub>2</sub> in air at 38°C. It is, therefore, necessary before any discussion of the changes in the different acids encountered in disease can be attempted, to define the effects of CO<sub>2</sub> tension on the various acids of the plasma.

Table I shows the difference in CO<sub>2</sub>, Cl, and protein of nineteen specimens of blood caused by altering the CO<sub>2</sub> tension from 30 to 60 mm., the range of ordinary variation. Under these circumstances the cells may be expected to imbibe a certain amount of water from the plasma, the H $\bar{\text{C}}\text{O}_3$  of the plasma will increase, and the  $\bar{\text{C}}\text{I}$  will diminish. These reactions may be considered as established qualitatively and if they do not appear in these experiments it is reasonable to believe that the experimental procedure which has been described at length in the preceding section was not entirely satisfactory or at least not sufficiently accurate for the purposes for which it was employed.



On this basis the method for the determination of cell volume is evidently deficient. Five out of nineteen times the hematocrit indicates a diminution of the cell volume as the  $\text{CO}_2$  increases. Presumably errors in the method will have an equal tendency to occur in either direction and the average degree of change obtained by adding all the positive and negative deflections separately, subtracting the latter from the former, and dividing by the total number of observations, will give some idea of the extent of cell volume changes induced by the given carbon dioxide alteration. In this series the average is 0.6 volumes per cent. In another series of eleven similar experiments the cell volume measurement fell only once as  $\text{CO}_2$  increased. The average change of cell volume was 1.0 per cent. For the total thirty experiments the cells increased in volume by an average of 0.76 volumes per cent. The error involved in the hematocrit method was estimated in the discussion of error in a previous paper as not much less than 1 volume per cent.

The chief importance of knowing the cell volume change in these experiments is that it offers a means of estimating the extent to which the proteins and base are concentrated. Protein was directly determined in only the 30 mm. sample of each experiment and the protein concentration of the corresponding 60 mm. sample was estimated on the assumption that protein did not cross the cell membrane and that changes of plasma volume in each case resulted in an inverse proportional change in the plasma protein concentration. If the cells swell it follows that the plasma proteins increase as the  $\text{CO}_2$  tension rises. The effect of this change on the acid value of the protein is not great, amounting in no instance to more than 0.6 mm. It is more than offset by the diminution of base-combining value induced by reduction of the plasma pH. The average reduction of the base-combining power of protein in these experiments amounted to 0.8 mm.

The change in  $\text{H}\bar{\text{C}}\text{O}_3$  is considerable, averaging 5.2 mm. For reasons that have already been considered (3), the extent of this change bears a general direct relation to the concentration of hemoglobin or the volume of cells in the blood.

In one instance Cl apparently increased; in the other eighteen the expected diminution occurred. In only one instance, Case 15293, was the reduction sufficient to compensate for the increased acid value of  $\text{H}\bar{\text{C}}\text{O}_3$ . The average change of Cl was 2.1 mm. 40

per cent of the corresponding  $\text{H}\bar{\text{C}}\text{O}_3$  change. Although the chloride method is less accurate than the determination of  $\text{CO}_2$  and the individual chloride estimations cannot, therefore, be interpreted too rigidly, this average probably represents more or less accurately the relative magnitude of the chloride and bicarbonate variations. The extent of the bicarbonate change is mainly determined by the concentration of hemoglobin or the volume of the cells of the blood. Cl does not appear to be influenced by the same factors. No definite conclusion can be drawn, however, from the apparent dissociation of the two acids in these experiments because the changes of Cl are so small. It may be that studies of chloride by a more delicate method or at more divergent  $\text{CO}_2$  tensions will give more consistent results.

The purpose of the present analysis is to ascertain the effect of a 30 mm. change of  $\text{CO}_2$  tension on the "total" acid content of the plasma. The results of the experiments indicate that the sum  $\text{H}\bar{\text{C}}\text{O}_3 + \bar{\text{Cl}} + \text{protein}$ , averages 2.2 mm more at 60 mm. of  $\text{CO}_2$  tension than it does at 30 mm. with a variation of from +5.1 to -7.0 mm. Phosphate has been ignored. If inorganic phosphorus appears only in the plasma, it will be influenced by changes in  $\text{CO}_2$  tension only in so far as the latter affect the volume of the plasma. In this case the changes in phosphate must be entirely negligible. Such an increase of acids cannot, of course, occur without an equivalent increase of base because the sum of the acid must equal that of the base. If the alkaline cations do not pass across the cell membrane the total amount of base in the plasma cannot change. The concentration of base will, however, increase by virtue of the reduction of plasma volume that results from the transfer of water to the cells. In the last column of Table I the change of base due to this factor has been estimated on the assumption that the original base concentration was 150 mm. The average increase of base calculated in this manner amounts to 1.7 mm. This accounts for all but 0.5 mm of the acid change. The deficit is insignificant in view of the assumed base value, and the errors in the determination of plasma volume, the base-combining power of protein, and other factors involved in the calculation.<sup>1</sup>

<sup>1</sup> This leads naturally to the conclusion reached by Doisy and Beckmann (1) that the shift of Cl and water between the cells and plasma accounts for all or practically all of the bicarbonate which is transferred in the opposite direction.

*Effect on the Acidity of Plasma of Changing the CO<sub>2</sub> Tension from 30 to 60 Mm.*

Case No.	Plasma.									
	Oxygen capacity.	Cell volume.	Plasma volume.	Protein.*	CO <sub>2</sub>	Cl	Acid, Columns 4+5+6	pH	CO <sub>2</sub> tension	Calculated base.
	(1)	(2)	(3)	(4)	(5)	(6)	(8)	(11)		
	vols. per cent	vols. per cent	vols. per cent	per cent	vols. per cent	mg. per 100 cc.	mm		mm.Hg	mm
5919	4.95	11.1	88.9	5.58	31.0	378	128.5	7.23	30	150.0
		11.4	88.6	*	39.9	369	128.2	7.02	60	150.5
10400	12.4	30.3	69.7	5.70	25.7	351	118.5	7.14	30	150.0
		29.3	70.7		36.5	350	121.3	6.98	60	147.9
26343	15.9	38.6	61.4	7.01	54.3	354	135.8	7.49	30	150.0
		38.0	62.0		67.1	351	138.6	7.27	60	148.5
18292	16.1	40.0	60.0	7.00	49.6	366	136.8	7.45	30	150.0
		41.2	58.8		62.9	355	138.0	7.24	60	153.0
12862	16.1	33.6	66.4	5.89	38.4	441	150.5	7.33	30	150.0
		34.2	65.8		50.5	438	153.4	7.13	60	151.3
10454	16.2	38.5	61.5	4.65	28.5	362	121.4	7.19	30	150.0
		38.7	61.3		40.1	359	124.1	7.02	60	150.5
13132	16.5	35.8	64.2	7.42	47.8	374	139.1	7.43	30	150.0
		38.0	62.0		61.5	370	142.4	7.23	60	155.3
10674	17.1	37.8	62.2	6.98	51.9	383	142.9	7.47	30	150.0
		37.5	62.5		66.2	376	145.2	7.26	60	149.2
10495	17.9	35.2	64.8	6.70	51.3	382	141.6	7.46	30	150.0
		36.2	63.8		64.9	381	145.8	7.25	60	152.4
5241	18.3	38.8	61.2	6.17	46.7	346	128.4	7.42	30	150.0
		38.4	61.6		57.9	339	129.4	7.20	60	149.0
9426	18.9	43.3	56.7	6.67	57.8	345	134.4	7.52	30	150.0
		43.1	56.9		73.4	339	137.6	7.31	60	149.5
22158	20.7	46.2	53.8	7.81	46.9	354	133.6	7.42	30	150.0
		45.9	54.1		61.1	357	138.7	7.22	60	149.1
5105	21.3	45.4	54.6	6.91	48.7	397	145.0	7.44	30	150.0
		46.1	53.9		64.0	385	147.0	7.25	60	152.0
4729	21.3	45.7	54.3	7.61	46.4	388	142.7	7.42	30	150.0
		46.7	53.3		61.7	385	146.9	7.23	60	152.8
12016	22.4	46.6	53.4	7.71	47.3	382	141.5	7.42	30	150.0
		47.6	52.4		63.6	371	144.0	7.24	60	152.8
3814	21.3	43.3	56.7	4.63	57.9	389	143.1	7.52	30	150.0
		44.0	56.0		73.6	378	145.4	7.31	60	151.8
3807	23.6	52.5	47.5	6.26	60.4	354	137.3	7.53	30	150.0
		54.1	45.9		77.7	343	140.5	7.34	60	155.2
	21.8	45.7	54.3	7.05	65.7	353	141.1	7.57	30	150.0
		47.5	52.5		79.9	346	143.9	7.35	60	155.0
15293	20.0	44.2	55.8	5.90	47.9	407	135.5	7.43	30	150.0
		46.3	53.7		63.2	364	138.5	7.23	60	155.9
Average change....	+0.6	-0.6		mm -0.8	mm +5.2	mm -2.2	mm +2.2	mm -0.20		mm +1.7

\* Protein was determined at 30 mm. only. The protein at 60 mm. was calculated from the 30 mm. value on the assumption that the alteration of cell volume was entirely due to water exchange and that the proteins would

The effect of oxygen on the acids has not been determined; but, from the fact that for a given change in oxygen the  $\text{CO}_2$  capacity of blood is altered about one-third as much as the oxygen content of the blood, one can argue that the effect on  $\text{Cl}$  and "total" acid will be reduced proportionately.

The effect of the transfer of chloride to cells in response to increase of  $\text{CO}_2$  tension is to liberate base in the plasma to combine with carbonic acid and thus to increase the buffer value of plasma. The chloride and bicarbonate ions which pass into the cells rob the proteins of the cells of an equivalent amount of base. The high concentration of protein in the cells provides an ample excess of base for this purpose. Practically the buffers of the cells are rendered available to the plasma. It is reasonably certain that similar reactions must occur between the plasma and the tissues and between the tissue cells and tissue fluids. These reactions do not lend themselves as readily to experimental investigation. Nevertheless, by means of such investigation alone can one hope to find an explanation of the electrolyte disturbances encountered in disease.

#### SUMMARY AND CONCLUSIONS.

The sum of the base-combining powers of the acids,  $\text{H}\bar{\text{C}}\text{O}_3 + \bar{\text{Cl}} + \text{protein}$  of the plasma of oxygenated blood increases on the average about 2 millimols when the  $\text{CO}_2$  tension of the blood is increased from 30 to 60 mm. at  $38^\circ\text{C}$ .

1. In this change  $\text{H}\bar{\text{C}}\text{O}_3$  increases 5 millimols. The extent of the increase is determined chiefly by the concentration of hemoglobin or the volume of the cells of the blood.

2. The cells swell slightly, diminishing the volume of the plasma and consequently augmenting the concentration and base-combining power of protein and, to a lesser extent, of phosphate. This is a little more than offset by the diminution of the acid value of protein brought about by reduction of pH. The average change of plasma volume amounts to  $-0.6$  volumes per cent, while the base-combining power of the proteins diminishes about 0.8 mm.

3.  $\bar{\text{Cl}}$  decreases by about 2 millimols, compensating for a little less than one-half of the  $\text{H}\bar{\text{C}}\text{O}_3$  change.

4. Because base does not traverse the cell membrane the loss of water from plasma to cells results in a concentration of base that neutralizes the excess acid.

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## **TOTAL ACID-BASE EQUILIBRIUM OF PLASMA IN HEALTH AND DISEASE.**

### **III. THE DIFFERENCES BETWEEN ARTERIAL AND VENOUS BLOOD.**

By JOHN P. PETERS, HAROLD A. BULGER, AND ANNA J. EISENMAN.

*(From the Department of Internal Medicine of Yale University and the  
Medical Service of the New Haven Hospital, New Haven.)*

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In the past the assumption has been generally made that, as regards electrolytes, arterial and venous blood differ only in respect to the amount of carbon dioxide and oxygen which they carry and that, if both are exposed to the same concentration of these two gases, they will prove to be identical. The first hint that this was not the case was provided by experiments in which arterial and venous blood were simultaneously analyzed for oxygen and carbon dioxide. If these blood gases alone were altered one would expect that respiratory quotients obtained by dividing the difference in the carbon dioxide content of the arterial and venous blood samples by the difference in oxygen would agree with respiratory quotients obtained from respiratory metabolism experiments. On the contrary, results reported by observers employing various technical procedures gave absurd respiratory quotients (12, 6, 9, 8, 2). In the discussion of their experiments Peters, Barr, and Rule (9) suggested that these improper respiratory quotients must be considered as indications of errors in the technical procedures employed. This suggestion was taken up by Doisy and Beckmann (4) who used respiratory quotients as criteria of the accuracy of certain experiments carried out on dog blood. For the technique which they employed they claimed an accuracy that should have insured success. Nevertheless they were compelled to discard several experiments because improper respiratory quotients were obtained.

**TABLE I.**  
*Difference between the Acids of Arterial and Venous Blood.*

[illegible]

19.3	39.7	7.47	61.6	72.5	287	360	7.46*	13.3	99	0	100.0	98.4	45.2	177	27.2	19.3	7.9	80	0	60.5	19.5	133.9	69.9	79.8	27.4	V	cont.				
15.3	32.9		44.1	51.2		364	7.45*	11.8				29.8			19.7	15.3	4.4			68.7	125.2		84.0		A	"					
15.4	33.8		45.9	53.0	309	378	7.49*	10.5	99	4	102.0	99.0	32.0	174	20.4	15.7	4.7	86.5	70.7	16	0	130	2	63.4	86.4	20.7	V				
18668	10.0	22.9		66.8	73.5	271	321	7.56*	7.8				44.6	103	29.8	25.3	4.5	76.4	69.7	6.7	123	2	49.0	95.0	11.2	A	"				
10.5	23.6		69.6	77.1	276	319	7.51*	3.7	96.2	99.1	95.4	45.4	139	29.9	25.3	4.6	74.8	66.0	8.8	124	2	59.4	91.3	13.4	V	"					
18112	18.1	38.2		58.3	69.1	326	381	7.41*	13.7			40.8		239	26.0	19.1	6.9	91.8	66.2	25.6	137	9	85.4	85.3	32.4	A	"				
17.7	36.5		59.7	69.7	322	390	7.35*	13.2	102.2	97.6	105.0	42.2	205	27.2	20.2	7.0	92.8	71.3	21.5	141.0	76.4	91.5	28.5	V	"						
16.5	36.7		57.9	66.4	303	360	7.47*	14.5				43.3	212	25.8	18.8	7.0	85.3	64.2	21.1	131.1	179	0	83	0	28.1	A	"				
17.2	37.7	13.6	1.5	71.3	300	360	7.39*	9.0	95.9	98.7	94.3	45.4	202	26.3	19.0	7.3	81.2	60.6	20.6	133	3	77.1	179.6	27.9	V	"					
16.2	35.0		55.9	64.1	319	358	7.42*	12.6				40.6	246	25.0	18.6	6.4	89.8	65.6	24.2	129	4	87.5	84	2	30.6	A	"				
16.2	35.0		60.1	69.2	307	365	7.37*	8.0	100.0	100.0	100.0	43.2	200	26.8	20.1	6.7	86.5	66.8	19.7	133.6	75.5	88.9	26.4	V	"						
18081	19.3	39.1	6.5	4.5	66.5	293	361	7.45				38.6		186	24.8	18.1	6.7	82.4	61.9	20.5	131.3	69.7	80	0	27.2	A	cap.				
20.3	42.1		52.5	64.7	294	367	7.44		95.1	102.2	90.5	35.6	193	22.3	15.9	6.4	78.7	56.9	21.8	132.0	70.3	72	8	28.2	V	"					

\* pH values followed by \* are 0.0 to 0.2 too high because the indicator solution used for these determinations was too acid. Cf. discussion of Methods (11).

A and V in the last column indicate arterial and venous blood respectively; cont. that the specimen was analyzed as drawn; cap. that it was brought into equilibrium with 40 mm. of CO<sub>2</sub> in air at 38°C. before analysis.



With the development of an accurate technique (1, 7) it seemed reasonable to expect that proper respiratory quotients would be secured with ease and regularity. We therefore set out to study arterial and venous blood with bright hopes. The results were quite as disappointing as ever, as evidenced by the experiments in Table I. This time, however, the authors had sufficient confidence in their technique to feel certain that the cause of the abnormal respiratory quotients must be sought elsewhere. Shortly before this Dautrebande, Davies, and Meakins (3) had pointed out that in pathologic conditions arterial and venous blood specimens might be entirely different in character and that oxygen- and carbon dioxide-carrying powers were among the properties affected. The precaution had therefore been taken to determine oxygen capacity and cell volume as well as oxygen content. The results confirmed the observations of Dautrebande *et al.* Since this time Fraser, Graham, and Hilton (5) have added further evidence to the same effect.

Even in the passage of blood from arteries to veins through the tissues sufficient time elapses to permit the loss or accession of considerable amounts of water and electrolytes and to alter the whole behavior of the blood to carbon dioxide and oxygen. These changes must have an effect on the respiratory quotients in the blood. The amount of carbon dioxide and oxygen given up or absorbed by the blood in the lungs and the tissues will be determined by the capacity of the blood to carry these gases. If the oxygen and carbon dioxide absorption curves of the blood change as it traverses the tissues, the respiratory quotient can no longer be calculated by a mere process of subtraction. The true respiratory quotient can be obtained from the blood by determining the amount of  $\text{CO}_2$  that will be given off and the amount of oxygen that will be absorbed when the venous blood is exposed to the tensions of  $\text{CO}_2$  and oxygen that exist in arterial blood. For such calculations one must know the carbon dioxide absorption curves of both arterial and venous blood. The question has been considered in detail by one of us and illustrated by an experimental example (10).

The last experiment in Table I (Case 18081) illustrates the difference that may occur even in an individual who presents no evidence of serious circulatory disturbance. In this case the

CO<sub>2</sub> capacity of the venous blood at 40 mm. of CO<sub>2</sub> tension proved to be 3 volumes per cent lower than that of the arterial blood. When it is considered that the technique employed in these experiments involves an error of only  $\pm 0.2$  volumes per cent it becomes obvious that this change represents a real difference between the behavior of the two bloods to carbon dioxide. Such a difference could only be brought about by an alteration of some of the other components of the blood. That these other components are altered is evident from the differences in oxygen capacity and whole blood chlorides. These changes are distinguished from those considered in the preceding paper by the fact that they represent not only alterations in the distribution of the chemical components of blood between the two phases of the blood itself, but an actual loss or accession of certain substances to or from the tissues through which the blood has passed.

In Table I are presented the results of fifteen simultaneous examinations of the acids of arterial and venous blood of nine patients made by the methods described in the first paper of this series (11). In all but one the blood was examined as it was drawn, without contact with air. In the last experiment (Case 18081) it was first brought into equilibrium with 40 mm. of CO<sub>2</sub> in air at 38°C. The first 9 columns give the actual analytical results, the following columns certain calculations derived from the analytical data.

In Column 10 is shown the relative volume of the venous specimen of blood as compared with the arterial, on the assumption that changes of hemoglobin are entirely due to alterations of blood volume. This assumption is not necessarily correct. In fact, there is considerable evidence in other experiments that factors other than the hydration of the blood affect hemoglobin. It is quite probable that cells may become segregated in the tissue capillaries at times, to be released under other circumstances. In the experiment on Case 18401 calculations based on oxygen capacity and cell volume indicate dilution of the plasma, although the plasma proteins have become more concentrated in their course through the tissues. The value in Column 10 does indicate the volume of the venous blood that contains the same amount of hemoglobin as the arterial blood. This is obtained by the following equation:

$$\frac{100 H_a}{H_v} = \text{relative blood volume} \quad (1)$$

where  $H_a$  and  $H_v$  represent the oxygen capacities of arterial and venous blood respectively.

In Column 11 is given the volume of venous cells that contains the same amount of hemoglobin as the arterial blood, derived by means of the following equation:

$$\frac{H_a(100 - c_v)}{H_v} \quad \text{relative cell volume} \quad (2)$$

where  $c_a$  and  $c_v$  represent the cell volumes of arterial and venous blood respectively.

The relative plasma volume, Column 12, is obtained by equation 6.

$$\frac{100 - c_a}{H(100 - c_v)} = \text{relative plasma volume} \quad (3)$$

$$H_v$$

The concentration of  $\text{CO}_2$  and Cl in the cells is calculated from the concentrations of the same substances in blood and plasma and the cell volume in the usual manner.

$$\frac{B - P(1.00 - C)}{C} = \text{concentration in cells} \quad (4)$$

where  $B$  and  $P$  represent the concentration of the given substance in blood and plasma respectively and  $C$  = the volume of cells in 1 cc. of blood.

Columns 21 and 22 give the concentrations of  $\text{CO}_2 + \text{Cl}$  in millimols in cells and in plasma.

In Columns 15, 16, 17; 18, 19, 20; and 23 and 24 are given the actual millimols of  $\text{CO}_2$ , Cl, and  $\text{CO}_2 + \text{Cl}$  in the original unit of blood cells or plasma before and after it has traversed the tissues. These values are obtained by multiplying the concentration of the different substances by the volume of the components. Thus for arterial blood:

$1.00 B$  = the actual amount of a given substance in arterial whole blood and

$\frac{H_a B}{H_v}$  = the amount of the same substance in the same unit volume of blood that has become venous. (5)

In the same way the amount in the cells is expressed by  $c_a C$  for arterial blood, where  $C$  is the concentration of the substance in the cells, and by

$$\frac{H_a c_v C}{H_v} \quad \text{for venous blood} \quad (6)$$

The amount in arterial plasma is  $(1.00 - c) P$  and in venous plasma

$$\frac{H_a (1 - c_v) P}{H_v} \quad (7)$$

No extended discussion of the table is warranted because the variations in all constituents are extreme and do not even show any consistency in direction. No clue to the cause of these inconsistencies appears in the experiments themselves. In some instances experimental errors may be responsible for part of the confusion because the observed changes are hardly great enough to determine with accuracy. There is not, however, any good reason for believing that more accurate methods would alter the situation in any fundamental manner. If only those experiments are retained in which the changes are large enough to be unmistakable the variations in the different elements analyzed remain just as capriciously inexplicable.

In two respects, only, are the experiments as they stand, of any great value. First, because they emphasize again the fallacy of assuming that arterial and venous blood may be used interchangeably in studies of the respiratory functions and electrolyte equilibria of the blood. Secondly, because they permit an estimation of the difference in concentration of chloride and bicarbonate, the two chief acids, in the transformation from arterial to venous blood. From Column 21 it may be seen that this difference usually amounts to about 2.5 millimols, but may be as much as 5 millimols. The venous blood contains more acid than the arterial in ten of fourteen experiments. In no instance did the arterial acid exceed the venous by a considerable amount, the highest negative value of *venous acid* — *arterial acid* being only 2.6 millimols. It is fair to add that the patients selected for this study were chosen because it seemed likely that they would present maximum *venous-arterial* differences. Most of them were suffering from severe cardiac decompensation.

Although it is impossible to ascribe the variable changes of the separate blood constituents to definite causes, it is probable that they are all due to reactions between the tissues and the plasma quite similar to those that govern the exchange between blood cells and plasma *in vitro* in response to alterations of carbon dioxide or other electrolytes. The alterations that occur in the tissues are, however, of greater complexity than any that can be reproduced in the test-tube and, consequently, produce a more complex disturbance of the blood electrolytes.

## SUMMARY AND CONCLUSIONS.

1. The general conception that arterial and venous blood differ as regards electrolyte equilibria only in so far as they contain more or less carbon dioxide and oxygen is erroneous. Determinations of oxygen capacity, cell volume, plasma proteins, and whole blood chlorides show that arterial and venous blood may contain also different amounts of water and chloride. In keeping with this is the fact that carbon dioxide absorption curves of the two bloods also may differ.

2. This explains the fact that attempts to obtain proper respiratory quotients by simple comparison of the differences in oxygen and carbon dioxide contents of arterial and venous blood have usually failed.

3. The changes that occur while the blood is traversing the tissues affect the separate components to different degrees and in different directions; the reasons for these apparent inconsistencies have not, as yet, been determined.

4. The end-result on the plasma acids,  $\text{H}\bar{\text{C}}\text{O}_3 + \bar{\text{C}}\text{l}$  of the transformation from arterial to venous blood is an average alteration of 2.5 millimols, usually an increase. The maximum changes encountered in fourteen examinations of nine patients, selected because it seemed likely that they would present large *venous-arterial* differences, were +5 and -2.5 millimols.

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## TOTAL ACID-BASE EQUILIBRIUM OF PLASMA IN HEALTH AND DISEASE.

### IV. THE EFFECTS OF STASIS, EXERCISE, HYPERPNEA, AND ANOXEMIA; AND THE CAUSES OF TETANY.

By JOHN P. PETERS, HAROLD A. BULGER, ANNA J. EISENMAN,  
AND CARTER LEE.

(*From the Department of Internal Medicine of Yale University and the Medical Service of the New Haven Hospital, New Haven.*)

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In order to study in detail the effect on the total acid-base equilibrium of certain conditions that might be expected to influence the water content and the electrolyte distribution of the blood a series of special experiments was carried out on the single subject JP and compared with individual studies of other normal persons and patients. The general methods of procedure which were followed in these experiments have been described in the first paper of this series (21).

#### *Effect of Venous Stasis.*

The first three experiments on JP (see protocols and Table I) illustrate the effects of prolonged venous stasis.

*Experiment of March 11, 1923. Subject JP (See Protocols of Paper I).—*In all the experiments of this series blood was withdrawn about an hour or an hour and a half after a light breakfast. For 20 or 30 minutes before the venipunctures the subject sat quietly in a chair with one or both hands and forearms immersed in warm water.

A tourniquet was applied to the arm with sufficient force to obstruct the venous return without obliterating the radial pulse. The pressure was maintained until the forearm and hand had developed a marbled cyanotic appearance and were quite painful. A specimen of blood was then withdrawn from the vein of the arm without disturbing the tourniquet. Another sample of venous blood was simultaneously secured from the other arm, without stasis. In the experiments of March 18 and April 18 the same procedure was followed. The experiment of May 13 was similar to those of March 11 and 18 and April 18, except that the blood samples were withdrawn immediately after the tourniquet had been removed. For data of these experiments see Table I.



TABLE I.  
Effect of Venous Stasis on the Total Acid-Base Equilibrium of Plasma.

Sub- ject.	Date.	Oxy- gen capac- ity.	Cell vol- ume.	Plasma.						Nature and treatment of blood.	Remarks.
				Pro- tein.	CO <sub>2</sub>	Cl	Total acid.	Total base.	Organ- ic acid.	pH	
		(1)	(2)	(4)	(5)	(6)	(8)	(9)	(10)	(11)	(13)
	1984	vols. per cent	vols. per cent	per cent	vols. per cent	mg. per 100 cc.	mm	mm	mm		
JP	Mar. 11	18.6	42.7	6.71	60.8	370	143.5			7.34	V. cont.
	"	22.5	51.2	9.13	65.4	349	143.1			7.29	"
Apr. 18		18.4	41.4	7.40	56.3					7.40	" cap.
		22.7	52.1	8.95	49.8					7.34	"
May 13		18.5	42.8		54.6	368	140.0	155.1	15.1	7.36	"
		22.8	53.9					156.0			"
		19.1	45.2	6.34	54.3	366	139.0	158.8	19.8	7.36	"
		20.5	47.9	7.54	52.5	362	139.2	158.7	19.5	7.34	"

In this and all succeeding tables A and V in Column 13 indicate arterial and venous blood respectively; *cont.*, that the specimen was analyzed as drawn; *cap.*, that it was brought into equilibrium with 40 mm. of CO<sub>2</sub> in air at 38°C. before analysis.

Dautrebande, Davies, and Meakins (8) have shown that such a procedure results in the passage of water and electrolytes to the tissues from the blood in consequence of which the blood becomes dehydrated. This is amply confirmed by the experiments of March 11, March 18, and April 18, if plasma protein, hemoglobin, and cell volume can be interpreted as an indication of the water concentration of the blood. The plasma on both occasions lost some 30 per cent of its water. Under these circumstances both protein and bicarbonate rose, while chloride fell. The fall in chloride was sufficient to compensate exactly the increase in the other elements. The "total" acid of March 11 did not change as much as a millimol and the total base of April 18 increased only about 1 millimol.

In these experiments an abnormal acid load was placed on the blood, first of all by the increase of protein and secondly by the accumulation of carbon dioxide. Under ordinary circumstances the organism might have responded by increasing the blood flow and the respirations. By these means the load of  $\text{CO}_2$  could have been removed and base freed for combination with the additional protein. The presence of the tourniquet precluded such a reaction and forced the body to meet the emergency in some other manner. It is clear from the experiment of March 18 that, under these circumstances, the carbon dioxide absorption curve falls. It is possible, then, for the organism to reduce the level of the carbon dioxide capacity of the blood without the mediation of the respiratory mechanism. The importance of this fact from the standpoint of maintaining the reaction of the blood at a constant level and facilitating the escape of carbon dioxide when the blood again has access to the lungs has been discussed in another connection (20). The constancy of base and total acid in the different experiments renders it unlikely that abnormal acids have entered the blood in appreciable amounts and forces the conclusion that bicarbonate has yielded base to the proteins.<sup>1</sup> The production of  $\text{CO}_2$  by the tissues, however, ex-

<sup>1</sup> It may be objected that the acid values for protein are so uncertain that this argument is invalid. In that case one must suppose that the increase of protein took up only a fraction of the base freed by bicarbonate and chloride and the remainder combined with organic acid. The constancy of the acid values before and after stasis must then be looked upon

ceeds the drop in bicarbonate. The only recourse then, is for chloride to give up some of its base. That such weak acids as proteins and bicarbonate should be able to displace as strong an acid as hydrochloric at first seems incomprehensible. The reaction, however, has long been known to occur in the equilibria between plasma and blood cells.

The experiment of May 13 differed somewhat from the three preceding ones in that the second sample of blood was withdrawn after the tourniquet had been removed. It was hoped that it might show a reversal of the process observed in the earlier experiments; *i.e.*, that the blood from the arm just relieved from stasis would be more dilute than the normal blood. In this respect it proved a disappointment, possibly because the venipuncture followed too closely the removal of the tourniquet. The changes observed are less marked than those of the previous studies, but are of the same nature. Evidently the effects of stasis on the tissues persist for a certain time after stasis is relieved. Whether a reversal of the phenomena could be demonstrated at a later period was not determined.

Studies of the oxygen content of the stasis blood revealed a high grade of anoxemia. Under these circumstances a certain amount of lactic acid might have been expected to accumulate in the blood. If it did it appeared in undemonstrable quantities. The muscles were, of course, kept at rest during the experiment, so that the lactic acid production was presumably quite small.

### *Effects of Exercise.*

The effect of exercise on JP (see protocols and Table II) offers a striking contrast to that of simple venous stasis.

*Experiments of March 27 and April 3, 1924, and June 5, 1925. Subject JP.*—These experiments were carried out about an hour or an hour and a half after a light breakfast. For 20 or 30 minutes before the venipunctures the subject sat quietly in a chair with one or both hands and forearms immersed in hot water. With the forearm supported on a table the hand and wrist

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as a mere coincidence. These coincidences throughout the whole work recur with such frequency that one can hardly escape the impression that the protein calculations are not greatly in error, at least from a relative point of view.

TABLE II.  
*Effect of Exercise on the Total Acid-Base Equilibrium of Plasma.*

Sub- ject.	Date.	Oxy- gen capac- ity.	Cell vol- ume.	Plasma.						Nature and treatment of blood.	Remarks.
				Pro- tein	CO <sub>2</sub>	Cl	Inor- ganic P.	Total acid.	Total base.	pH	
		(1)	(2)	(4)	(5)	(6)	(7)	(8)	(9)	(11)	(13)
		vols. per cent	vols. per cent	per cent	vols. per cent	mg. per 100 cc.	mg. per 100 cc.	mm	mm		
JP	Apr. 3, 1924	18.3	38.1	6.41	57.0	357		137.8		7.37	V. cont.
		18.7	41.4	6.48	62.2	357	4.0	138.3	148.4	7.20	"
	June 5, 1925						4.4		162.6		"
											"
	Mar. 27, 1924	18.2	42.6	6.43	51.5	380		141.8		7.33	" cap.
		18.7	43.9		45.6	381		137.5		7.28	"
	Jan. 22, 1923	21.0	42.9	6.72	55.4	390		147.1		7.37	V. cap.
		22.0	42.9	7.62	42.3	380		139.3		7.24	"
											Without stasis.
											After stasis and exercise.

were alternately flexed and extended with a weight of about 10 pounds suspended from the finger tips, until the exercise had produced a state of painful fatigue. At this point, while the exercise was continued, blood was drawn synchronously, without stasis, from the veins of both arms.

*Experiments of January 22, 1923. Subject JP.*—The first sample of blood was withdrawn about an hour after breakfast, without stasis, but without any preliminary rest or preparation. Immediately after the blood had been obtained a tourniquet was applied to the arm with sufficient force to obstruct the venous return without obliterating the arterial pulse. The hand was then opened and closed rapidly and forcibly until the exercise became quite painful, when the second specimen was drawn while the tourniquet was still in place and while the exercise was continued.

In the first place exercise produces little change in the water content of the serum. The hemoglobin and cell volume do indicate a certain amount of concentration; but this is not reflected in the plasma proteins and may well be due to the liberation of additional blood cells from the capillaries as a result of the increased blood flow and vascular dilation produced by the exercise. From the experiment of March 27 it is clear that the  $\text{CO}_2$  capacity of the blood has diminished. This decrease is, however, more than offset by the increased carbon dioxide load, as is evidenced by the fact that the bicarbonate content of the blood (see experiment of April 3) is greater after exercise than before. Chloride in both experiments remains unaltered. The end-result is to increase the "total" acid slightly, from 137.8 to 138.3 mm.

Unless total base increases this "total" acid accession can only take place at the expense of the organic acid fraction. This is unlikely. In fact investigations of Barr and his associates (4) and of Lundsgaard and Möller (17) have demonstrated that exercise results in the discharge of lactic acid into the blood. This must augment the organic acid fraction of the serum. Base was not determined in the first experiments, but was estimated in a separate study carried out on June 5, 1925, under the same conditions as the earlier observations. As might have been expected base increased considerably. Attention must be called to the fact that on this occasion the preliminary sample of serum contained less base than any other specimens from the same subject. For this reason the experiment cannot be considered quantitatively comparable to the previous ones. Nevertheless it is probable that similar qualitative variations occurred in all of them.

The local reaction to short, fatiguing exercise, therefore, consists in the delivery to the blood of an excess of organic acid, presumably lactic. To meet this added acid load the blood responds to a slight extent by reducing the bicarbonate of the blood, but mainly by withdrawing base from the tissues. The plasma chlorides are not affected. It is worth remarking in passing that phosphate is not appreciably altered.

The experiment of January 22, 1923, illustrates the combined effects of stasis and exercise. This is, of course, comparable to exercise with insufficient oxygen. As might be expected under these circumstances, the reduction of bicarbonate is greater than that with stasis or exercise alone. Furthermore chloride is again forced to come to the aid of bicarbonate. "Total" acid, therefore, is greatly diminished. If this diminution is entirely due to the accumulation of lactic acid and protein the part played by each of these acids can be calculated. The total drop in acid = 7.8 millimols. Of this 0.9 millimols is caused by the protein and 6.9 millimols by lactic acid. Base was not determined. If base also rose this estimate of the amount of lactic acid produced is too small.

### *Effects of Voluntary Hyperpnea.*

That tetany could be produced by overventilation was first demonstrated by Grant and Goldman (12) and Collip and Backus (6), who ascribed the condition to alkalosis. The observation has since been confirmed by numerous workers and the associated electrolyte changes have been extensively investigated to determine the degree of change of the hydrogen ion concentration and the bicarbonate of the blood. In these studies indirect methods have, for the most part, been employed and the interpretation of observed phenomena has usually been open to question. The experiments of April 10 and 16 (see protocols and Table III) were planned to throw some light on certain features of over-ventilation tetany that had not, in the opinion of the authors, been satisfactorily settled by previous studies and for the solution of which the present procedure was peculiarly adapted.

*Experiments of April 10 and 16. Subject JP.*—About an hour after a light breakfast blood was withdrawn from the arm vein of the subject, who had been sitting at rest for 20 or 30 minutes with his hands and forearms

immersed in hot water. Immediately after the venipuncture he began to breathe as rapidly and forcibly as possible, giving his major attention to expediting and completing the expiratory phase of respiration. Symptoms came on very shortly, beginning with dizziness and marked tingling of the extremities. The hands became fixed in the position typical of carpopedal spasm, although the wrists were held in a position of adduction and extension. To change the position of the hands, while not impossible, was difficult and somewhat painful. The feet felt as if the shoes were too tight and as if the balls of the toes were pressed hard against the soles of the shoes. Although the subject did not lose consciousness he became distinctly dazed so that he did not discontinue overventilating at the end of the experiment until he had been told to stop three times. For some time afterwards there was almost complete apnea, but no marked cyanosis appeared. As soon as definite carpopedal spasm had developed a sample of blood was withdrawn, without stasis, from the same vein from which the preliminary sample had been obtained. Forced breathing was not discontinued until the blood had been secured. The whole experiment took only about 5 minutes.

Grant and Goldman (12), Davies, Haldane, and Kennaway (9), and others have reported the appearance of carpopedal spasm and other evidences of tetany only after hyperventilation had been carried on for 10 minutes or more. In so long a period certain secondary adjustments may take place that obscure the primary changes of tetany. To reduce the time necessary for the production of tetany as far as possible seemed desirable. It was conclusively demonstrated in two experiments on JP (see protocols) that a striking picture of tetany can be produced in 5 minutes or less by forced breathing alone, if the latter is performed in the proper manner. The ordinary individual when told to overventilate finds no difficulty in increasing the speed and depth of inspiration. Expiration, naturally a more passive movement, is usually unduly prolonged and is not carried to completion unless special emphasis is put upon it. By giving the major attention to accelerating and completing each expiration the effectiveness of the ventilation is greatly increased and the appearance of tetanic symptoms expedited. There can be no doubt from the description in the protocols that the symptoms and signs induced were those of tetany. The results of blood examinations appear in Table III.

The change of pH produced, only 0.2, was surprisingly small compared with that reported by other observers. Davies *et al.* (9), from comparison of alveolar  $\text{CO}_2$  and carbon dioxide absorp-





tion curves, estimated the change in one of their experiments as 0.38 of pH. It is more than possible that they underestimated the arterial  $\text{CO}_2$  tension. One cannot assume that the respired air during extreme overventilation comes into gaseous equilibrium with the arterial blood.

Y. Henderson (15) holds that the organism will respond to overventilation by reducing the carbon dioxide capacity of the blood, a reaction which would tend to maintain the pH of the blood at a constant level. There is considerable evidence (3) that such a reaction does occur during the adjustment to oxygen-want at high altitudes. If hyperventilation were prolonged sufficiently it is likely that compensation would be effected in a similar manner. In these experiments (see experiment of April 16) the carbon dioxide capacity remained practically unaltered. An excessive amount of carbon dioxide was, however, removed from the blood by reduction of the carbon dioxide tension. That chloride should diminish was quite unexpected; the diminution is, however, unmistakable in both experiments. Such a reduction of chloride with relatively little change in the other acids and with no alteration of base implies an increase of organic acid.

The nature of this organic acid was not investigated. Davies and his coworkers (9) detected acetone in the urine after over-ventilation, and after the administration of alkali. Adlersberg (1), who has confirmed their observations, has suggested that ketosis is one of the means by which the body is enabled to prevent alkalosis. The ketonuria, and presumably the ketonemia, reported by all these observers was extremely mild and could hardly account for more than a small part of the 5 millimols of abnormal organic acid found in the blood. It is not improbable that some or all of the remainder is lactic acid. The spasms of tetany certainly entail muscular activity, which must result in the production of lactic acid. On April 10 the oxygen content of the blood was determined before and during tetany. In spite of the vigorous respiration the tetany blood contained 3 volumes per cent less oxygen than the preliminary normal specimen. Tetany is, therefore, attended by a relative anoxemia which would promote the tendency to lactic acid formation. This anoxemia may be due to a retarded circulation or to an accelerated oxygen consumption. Macleod and Knapp (18) and

Anrep and Cannan (2) have both shown that lactic acid production is increased by alkalosis induced by the administration of bicarbonate. This, the latter observers think, is entirely due to the alkalosis which develops. If this is so, there is further reason for believing that part of the organic acid found in the serum after hyperventilation is lactic acid.

### *Effects of Anoxemia.*

If a large enough volume of air is rebreathed under conditions which preclude the accumulation of  $\text{CO}_2$  by individuals who are not peculiarly susceptible to the effects of oxygen-want, hyperventilation occurs after the oxygen concentration of the inspired air has fallen below a certain level and increases gradually as the oxygen continues to fall. The minute volume, however, does not usually increase to the point where breathing itself is distressing. The ventilation of JP, for instance, in the experiment of June 18 (Table IV and protocols), even at the end of the experiment when the air in the spirometers contained only about 7 per cent of oxygen and the subject was dazed, did not approach the volume attained by the same subject at other times when breathing high concentrations of carbon dioxide, nor was the anoxemia experiment attended by as much respiratory distress and effort.

*Experiment of June 11, 1925. Subject JP.*—About an hour after breakfast, without any preliminary preparation the subject, in a sitting position, rebreathed through soda lime from an ordinary Sanborn model Tissot spirometer which had been filled in advance with about 65 liters of air. The tubes connecting the subject with the spirometer were so long that a considerable dead space free from carbon dioxide, but probably of low oxygen tension, was interposed.

There was also continuous respiratory resistance. Consequently dyspnea began early and within little more than 5 minutes acute dyspnea had developed, with considerable cyanosis. Blood was taken from the arm vein just before the experiment and again when dyspnea had reached a maximum and the subject was extremely uncomfortable. Part of the first sample clotted, unfortunately, so that determinations of oxygen capacity, oxygen content, and cell volume could not be made. There was also a moderate amount of hemolysis in part of the second specimen.  $\text{CO}_2$ , Cl, and protein were estimated in the non-hemolyzed fractions of serum. Tingling of the extremities and dizziness appeared, but no signs of tetany developed.

*Experiment of June 18, 1925. Subject JP.*—About an hour and a half

TABLE IV.  
*Effect of Oxygen Deprivation on the Total Acid-Base Equilibrium of the Plasma.*

Subject.	Date.	(1) Oxygen capacity.	(2) Cell volume.	Plasma.							CO <sub>2</sub> content of separated plasma at CO <sub>2</sub> tension of:		CO <sub>2</sub> tension of blood	Oxygen content of blood.	Nature and treatment of blood.	Remarks.
				Protein.	CO <sub>2</sub>	Cl	Inorganic P.	Total acid.	Total base.	Organic acid.	pH	50 mm.	60 mm.			
		vols. per cent	vols. per cent	per cent	vols. per cent	mg. per 100 cc.	mg. per 100 cc.	mm	mm	mm	(11)	mm.	mm.	vols. per cent		
JP	1925 June 11			6.74	57.1	354	3.8	138.0	154.1	16.1	7.13	55.6	60.7	74.8	V. cont.	Before rebreathing.
				6.64	55.5	351	3.7	136.8	148.2	11.4	7.25	55.6	60.0	55.2	"	During (oxygen-want).
	"	18	17.1	43.6	36	365.0	4.3	143.2	158.4	15.2	7.29	59.4	65.7	55.3	"	Before rebreathing.
			16.1	42.6	36	374	2.5	142.8	156.0	13.2	7.35		60.8	43.1	"	During (oxygen-want).
HAB	"	26	(17.4)	43.97	36	365.9	3.48	4139.7	149.1	9.4	7.26	59.5	66.0	59.4	"	Before rebreathing.
			(17.5)	45.97	56	62.0	351	2.4	139.8	153.0	13.2	7.38	58.3	65.4	"	During (oxygen-want).

after breakfast, after a preliminary rest of more than 30 minutes and after both hands and forearms had been held immersed in hot water for about 20 minutes, the subject started rebreathing air from which the  $\text{CO}_2$  was continuously extracted with soda lime. This time a Benedict-Sanborn apparatus was connected in series with the Tissot spirometer in such a way that a continuous circulation was maintained throughout both spirometers by means of the fan of the Benedict apparatus. The apparatus was filled in advance with about 62 liters of room air, besides what the tubing and connections contained. With this apparatus the subject was able to continue rebreathing without extreme discomfort for about 28 minutes. After about 10 minutes the respirations became somewhat irregular at intervals and after 20 minutes assumed an irregular periodicity. From time to time after this slight tingling of the face and extremities developed. Towards the end of the experiment dyspnea became quite marked, dizziness and confusion developed, but not syncope, and the mouth relaxed so that some expiratory air, but no inspiratory air, escaped about the mouthpiece. Blood was withdrawn from the arm vein just before and at the very end of the experiment before the subject was disconnected from the apparatus. Parts of both samples of serum showed slight hemolysis.  $\text{CO}_2$ , Cl, and proteins were determined in the non-hemolyzed fractions of the two specimens of serum.

*Experiment of June 26. Subject HAB. (See Protocols of Paper I).—* This experiment was quite similar to that of June 18 on JP, except for the fact that only 40 liters of air were placed in the spirometer at the beginning. Rebreathing was continued for almost 30 minutes. Although the respiratory rate increased comparatively early and the minute volume gradually grew larger, even at the very end of the experiment, when the subject was strikingly cyanotic, the hyperpnea was quite moderate. At intervals the respirations were counted for a minute at a time and an attempt was made to estimate the tidal air by observing the excursion of the Benedict spirometer. The respiratory rate at different times was: in the 7th minute, 21; in the 11th, 21; in the 14th, 24; in the 19th, 22; and in the 24th, 23. The tidal air rose from less than 400 cc. in the 7th minute to over 700 cc. in the 24th. The ventilation continued to increase at a more rapid rate after this, but was not measured.

Although dyspnea never became distressing, the subject was distinctly uncomfortable by the end of the experiment and felt somewhat dazed. No signs or symptoms of tetany developed.

At the end of the experiment the spirometer air was found to contain only 5.60 per cent of oxygen and 1.19 per cent of  $\text{CO}_2$ . Through an error the stop-cock of one of the tubes was left open for some time before the samples were withdrawn. This probably caused no considerable error because the air in the spirometer was under slight positive pressure and the tube between the open stop-cock and the spirometer was 5 or 6 feet long. The oxygen figures must, however, be considered as maximum values and the  $\text{CO}_2$  as minimum. Apparently the soda-lime absorber was somewhat inefficient in this experiment.

In the first experiment pH was not determined. In the last two it was estimated by a gasometric technique devised by one of the authors.<sup>2</sup> This consists of separating the serum or plasma from blood, as drawn, without contact with air, in the usual way. The CO<sub>2</sub> content of this serum is determined. Two small samples are then saturated at 38°C. in micro-tonometers filled with CO<sub>2</sub> mixtures of different known tensions. These samples are also analyzed for CO<sub>2</sub>. By means of the following equation developed from the straight line formula of Peters (19) the CO<sub>2</sub> tension of the unknown blood may be calculated.

$$\log p_x = \log p_A - \frac{(\log p_A - \log p_B)(\log C_A - \log C_x)}{(\log C_A - \log C_B)} \quad (1)$$

where  $p$  and  $C$  represent the CO<sub>2</sub> tension in mm. of mercury and CO<sub>2</sub> content of the serum in volumes per cent respectively; the subscript  $x$  stands for the blood of unknown tension; and the subscripts A and B for the two samples saturated with known tensions of CO<sub>2</sub>.

The rebreathing experiments of June 18 on JP and of June 26 on HAB represent the effects of increasing, but always moderate, involuntary hyperpnea carried on for a comparatively long period of time and were planned to supplement the voluntary overventilation studies just described. Certain technical defects in the apparatus used in the experiment of June 11 modified the results by introducing complicating factors. In the June 11 observation obstruction to respiration and an excessive instrumental dead space resulted in the rapid production of symptoms that forced the early termination of the experiment. Although CO<sub>2</sub> and hydrogen ion concentration had fallen, chlorides had not changed. Base had diminished somewhat.

In the longer experiment of June 18 bicarbonate decreased considerably, far more than it did in any of the voluntary hyperpnea studies. The associated change of pH was, however, only

<sup>2</sup> A. J. Eisenman. This procedure has been employed in these experiments because of the uncertainty of colorimetric procedures. The Cullen procedure has been found by us and several other observers to give inaccurate results when applied to pathologic material because the temperature correction is variable. The more recent bicolorimetric method of Hastings, in which the pH is determined at 38°C., has been subjected to similar criticism by Austin and Stadie (personal communication). On the other hand, most observers agree that  $pK_1$  of plasma and serum is remarkably constant. It has seemed best, therefore, in the absence of facilities for electrometric determinations, to use a gasometric technique until the accuracy of the colorimetric procedure is established.

0.06 instead of the 0.20 of the preceding series. A greater change of reaction could only be prevented by the fixation by some other acid of a certain amount of the base previously bound by carbonic acid. The increase shows that this is what happened. Chloride, presumably derived from the tissues has almost exactly replaced the  $\text{CO}_2$  which was driven off by the overventilation and thus prevented a change of reaction that might have resulted in tetany.

The experiment of June 26 on HAB shows a different reaction. Attention has already been called to the fact that the subject developed little hyperpnea. This is especially noteworthy in view of the fact that the respiratory air contained an excess of  $\text{CO}_2$ . Evidently there is a large variation in the reaction of different individuals to oxygen-want. This is further illustrated in the oxygen contents of the blood of the two subjects. The final oxygen saturation of JP's blood was 70 per cent while HAB's fell to 27 per cent. Nevertheless HAB developed less hyperpnea than JP.

The reduction of  $\text{CO}_2$  bears a direct relation to the duration and intensity of the hyperpnea. It was least marked in the short experiment of June 11 and most marked in the experiment of June 18. The compensatory reactions also seem to be dependent on the same factors. In the experiment on HAB, therefore, since  $\text{CO}_2$  fell comparatively little, chloride was not significantly affected.

In both the last experiments protein rose, suggesting a concentration of the serum. In the study of HAB oxygen capacity and cell volume give similar indications, but the same is not true of JP, June 18. The increase of serum protein in the experiment of June 26 is almost exactly equivalent to the loss of  $\text{CO}_2$ .

The effects of the involuntary hyperpnea of oxygen-want then seem to depend on the duration and intensity of the overventilation produced. If the experiment is continued for 30 minutes the serum proteins increase slightly, the  $\text{CO}_2$  falls to a variable extent, and the pH increases moderately. If  $\text{CO}_2$  falls far enough to exceed the compensatory action of the protein increase, chloride is yielded by the tissues to combine with the base freed by carbonic acid. By this means the change of pH is minimized and, possibly, tetany is avoided.

Attention has already been called to the fact that base diminished slightly in the experiment of June 18. The change is so small that it is of doubtful significance. It may be that a diminution of base aids the chlorides in compensating for the fall of bicarbonate. In the experiment of June 26 on HAB base and organic acids both increase. The changes in the two elements are almost exactly equivalent. The degree of anoxemia attained in this instance may well have resulted in the appearance of lactic acid or some other acid metabolic products. This has led as it did in exercise, not to a displacement of other acids but to the transfer of base from the tissues. This is probably more strictly a response to anoxemia than is that exhibited by JP, which seems to have been determined rather by the overventilation.

The reduction of phosphate that appears in both the last experiments is as inexplicable as it was unexpected. As far as we know this has not yet been recognized as one of the effects of oxygen-want or overventilation.

#### *Hyperventilation without Tetany, a Case Study.*

Comparison of the voluntary hyperventilation and the oxygen-want experiments suggests that the differences in reaction are largely the results of the duration and intensity rather than the cause and nature of the hyperpnea which produced them. Thus short, comparatively mild overventilation on June 11 produced little change in the blood electrolytes; long, mild overventilation on June 18 increased pH slightly and reduced carbonic acid, but provoked little compensation; short, violent hyperpnea of the voluntary overventilation experiments resulted in a fall of carbonic acid, a striking increase of pH, and an appearance of organic acid that replaced chloride; the prolonged, moderate hyperpnea of June 18 reduced bicarbonate considerably, but the corresponding alteration of pH was prevented by the contribution of chloride from the tissues. In oxygen-want there are, however, certain elements that are lacking in voluntary hyperpnea, especially deficient oxidation of the tissues. It is quite possible that this modifies the results of overventilation and is the factor that causes the chlorides to replace the falling bicarbonate.

To settle the question it is, of course, necessary to study the

phenomena that follow long, moderate, voluntary hyperpnea. Experiments along this line were planned and will eventually be attempted. Meanwhile a patient with overventilation presented himself on the ward and afforded an opportunity to approach the subject indirectly.

*Case 28976.*—American, male, age 16, admitted to the hospital May 11, 1925. About 2 years before this he had a condition which was diagnosed as encephalitis lethargica, a period of 3 weeks during which he had fever and extreme lethargy. After this he was troubled with increasing weakness. In February, 1924, he developed shortness of breath of such severity that he was practically incapacitated. The next month he was sent to the hospital for examination and treatment. He presented at this time no evidence of localized lesions of the central nervous system. He had striking bilateral nystagmus. The respirations were much exaggerated during the greater part of the day, but he was able to hold his breath a long time and his breathing was comparatively normal when he was asleep. Blood Wassermann, spinal fluid, and other special examinations revealed no significant abnormalities. At the time of his second admission, May 11, 1925, the patient's condition was practically unchanged. He said that he had occasional attacks of tingling of the extremities and sometimes sudden seizures characterized by preliminary weakness and dragging down sensations that caused him to double up and sometimes resulted in vomiting. No more exact description of these attacks could be obtained and none occurred while he was in the hospital.

The patient appeared somewhat underdeveloped and rather thin. His color was good, his expression somewhat vacant. The eyes showed constant lateral nystagmus. The respirations were rapid and violent and he kept his mouth open constantly. The heart showed marked sinus arrhythmia. Further examination revealed no significant abnormalities.

Further study of the respiration showed that he could refrain from overventilation and even hold his breath for a considerable time by voluntary effort. During sleep and when his attention was diverted the breathing also diminished. There was a tendency for the abdomen and chest walls to move in opposite directions during the phases of respiration. This paradoxical motion became more marked as the breathing increased in violence. When he kept his mouth shut the overventilation diminished without causing him distress. The pulse, like the respirations, showed considerable variation in rate and was quite rapid. The temperature also showed more than the usual daily fluctuation, reaching 100°F. (rectal) almost every day. *Impression:* Encephalitis epidemica; late manifestations.

On May 15 a study of the respirations was made to determine how much of the overventilation was real and how much of it was only apparent. Before breakfast, and after the usual rest and preparation, the subject was connected with a Tissot spirometer in the usual manner employed for the determination of basal metabolism. The results of the examination are



given below. During the first run he breathed quite violently. The exact respiratory rate was not determined for each minute, but for 5 representative minutes it was 30, 28, 32, 30, and 32, with an average of 30.4 per minute. At the end of only 5.5 minutes the expiratory volume had already reached

TABLE V.  
*Respiratory Experiment of May 15, 1925. Subject 28976.*

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Weight 53.5 kilos. Height 169 cm. Surface area 1.61 sq.m.	
<i>First Run.</i>	
Total volume of air expired at 0°, 760 mm.....	100.3 litres
“ time of run.....	5.50 min.
CO <sub>2</sub> of expired air.....	1.88 per cent
O <sub>2</sub> “ “ “.....	19.17 “ “
Nitrogen of expired air.....	78.95 “ “
O <sub>2</sub> absorbed.....	1.76 “ “
CO <sub>2</sub> produced.....	1.85 “ “
Minute volume of air expired.....	18,240 cc.
Respiratory rate (average of rate for successive min., 30, 28, 32, 30, 32).....	30.4 per min.
O <sub>2</sub> absorbed per min.....	321 cc.
CO <sub>2</sub> produced “ “.....	338 “
R. Q.....	1.05
Total metabolism per hr. (R. Q. of 1.00 used for calculations)	94.4 cal.
Metabolism per sq.m. per hr., 58.6 cal.; 36 per cent above normal.	
<i>Second Run.</i>	
Total volume of air expired at 0°, 760 mm.....	109.0 liters
“ time of run.....	11.08 min.
CO <sub>2</sub> of expired air.....	1.68 per cent
O <sub>2</sub> “ “ “.....	18.75 “ “
Nitrogen of expired air.....	79.57 “ “
O <sub>2</sub> absorbed.....	2.33 “ “
CO <sub>2</sub> produced.....	1.65 “ “
Minute volume of air expired.....	9850 cc.
Respiratory rate (too irregular to determine).	
O <sub>2</sub> absorbed per min.....	229 cc.
CO <sub>2</sub> produced “ “.....	162 “
R. Q.....	0.71
Total metabolism per hr.....	64.6 cal.
Metabolism per sq.m. per hr., 40.1 “ ; 6.7 per cent below normal.	

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100.3 liters, giving a minute volume of 18.2 liters. The CO<sub>2</sub> concentration in this air was 1.88 per cent. Part of the respiration, that portion which merely exchanges the dead space air, is, of course, of no functional significance for respiration. This may become of some importance when the

respiratory rate is unduly increased. If the dead space in this case were 150 cc. with a respiratory rate of 30.4, the effective minute volume must have been  $18,240 - (30.4 \times 150) = 13,680$  cc. This is about three times as much as normal. The high respiratory quotient, 1.05, indicates overventilation, and the result of the basal metabolism determination, which proved to be 36 per cent above normal, is probably exaggerated by the same factor.

TABLE VI.

*Respiratory Experiment of May 22, 1925.*

Weight 54 kilos. Height 169 cm. Surface area 1.62 sq.m.

Time.	Respirations.	Volume of air expired.	Tidal air.	Effective minute volume.	Effective tidal air.
<i>min.</i>		<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
1	23	7400	322	3960	172
2	22	8300	377	4990	227
3	24	8700	417	6410	267
4	21	7000	333	3840	183
5	22	10,400	473	7110	323
6	25	10,400	416	6650	266
7	22	7000	318	3700	168
8	21	8700	414	5540	264
9	24	9600	400	6000	250
10.03	21	7800	371	4640	221
Total.. ....	225	85,300		50,750	
Average.....	22.4	8500	379	5060	229

CO<sub>2</sub> of expired air.....2.54 per cent

O<sub>2</sub> " " ".....18.05 " "

Nitrogen of expired air.....79.41 " "

O<sub>2</sub> absorbed.....3.01 " "

CO<sub>2</sub> produced .....2.51 " "

O<sub>2</sub> absorbed per min.....256 cc.

CO<sub>2</sub> produced " " .....213 "

R. Q.....0.83

Total metabolism per hr., 74.6 cal.

Metabolism per sq.m. per hr., 46 cal.; exactly normal.

A second run gave entirely different results. The patient was directed this time to close his eyes and attempt to go to sleep. Under these circumstances the respirations became very irregular. At times there was complete apnea for as much as 1.75 minutes. These periods were terminated abruptly and were succeeded by short spells of obvious dyspnea. The low respiratory quotient in this period, 0.71, suggests that he was compensating for the earlier dyspnea. In spite of this his minute volume is abnormally

large. It is probable, then that the increased respiration was not, at this moment, entirely due to psychic factors. The basal metabolism proved to be 6.7 per cent below normal. (The experimental data of the two respiratory experiments appear in Table V.) A little later blood was withdrawn from the brachial artery and used for an electrolyte study. During the arterial puncture the patient had another attack of extreme dyspnea. The results of this and subsequent blood analyses appear in Table VIII.

TABLE VII.

*Respiratory Experiment of May 29, 1925.*

Weight 54.2 kilos. Height 169 cm. Surface area 1.62 sq.m.

Time.	Respirations.	Volume of air expired.	Tidal air.	Effective minute volume.	Effective tidal air.
<i>min.</i>		<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>
1	28	7080	253	2880	103
2	26	7920	307	4070	157
3	23	10,630	462	7180	312
4	26	16,830	648	12,930	498
5	28	8860	316	4660	166
6	33	9750	295	4800	145
7	27	7970	295	3920	145
8	20	10,630	532	7630	382
9	28	11,510	411	7310	261
10 06	30	7970	266	3470	116
Total.....	269	100,050		59,710	
Average.....	26 7	9945	372	5935	222

CO<sub>2</sub> of expired air..... 2.40 per cent  
 O<sub>2</sub> " " ".....17.80 " "  
 Nitrogen of expired air.....79.73 " "  
 O<sub>2</sub> absorbed.....3.28 " "  
 CO<sub>2</sub> produced.....2.37 " "  
 O<sub>2</sub> absorbed per min.. ..326.5 cc.  
 CO<sub>2</sub> produced " " .....236 "  
 R. Q.....0.72  
 Total metabolism per hr., 92.2 cal.  
 Metabolism per sq. m. per hr., 56.8 cal.; 23.6 per cent above normal.

On May 22 a second study was made. Meanwhile the general condition of the patient had improved and his respirations were quieter. The respirations were studied as before; but the rate and volume at the end of each minute were noted separately. Arterial puncture was performed during the run. The site of puncture had been anesthetized in advance, the artery was rapidly and easily entered, and the subject said that he experienced no



pain at all. There was no objective evidence that he noticed the puncture. The respirations during the experiment were comparatively quiet and only slightly irregular. At times the left hand assumed a position that suggested carpopedal spasm, but could be voluntarily or passively relaxed. None of the other limbs was similarly affected and application of a bandage after the arterial puncture did not elicit any spasms. The results of the respiratory experiment appear in Table VI and the blood analysis in Table VIII.

This time the respirations were probably in general under the normal control (respiratory quotient 0.83). The metabolism was normal for one of his age and size. The rate and the minute volume were, however, still greater than normal. In view of these facts and the absence of any clear evidences of tetany or alkalosis it seemed justifiable to see what effect alkaline therapy would have on the symptoms. Accordingly 2 gm. of sodium bicarbonate were given on May 25, 5 gm. on May 26, 10 gm. on the 27th, 15 gm. on the 28th, and 5 gm. at 7 a.m. on the 29th. A third respiratory study was made at 8.30 a.m. May 29, 90 minutes after the last dose of bicarbonate.

The night following the second examination the patient got into a fist fight with another patient on the wards. The next day the respirations, which had been subsiding, returned to their former character. The administration of bicarbonate had no apparent effect on the respirations. Before the run he was overventilating wildly. After the mask was applied the breathing quieted down with surprising rapidity, with long periods of complete apnea. The quiet breathing continued throughout the 1st minute of the test. At the end of that time arterial puncture was begun. For some reason this proved difficult and painful, necessitating two punctures, and blood was not actually obtained until the 6th, 7th, and 8th minutes. The pain and difficulty is reflected in the respiratory record in Table VII. Over-ventilation was not, however, striking.

In spite of the abnormally large minute volume and the high respiratory rate, the low respiratory quotient suggests hypo- rather than hyperventilation, and the period of observation probably represented compensation for previous overventilation.

It is evident from the history and the respiratory studies that the patient was overventilating markedly for considerable periods; nevertheless he presented no symptoms of tetany nor any disposition to develop signs of tetany. Davies, Haldane, and Kennaway (9) and others found that alkalosis usually resulted in ketonuria. The urine in this case was examined repeatedly, but no acetone or diacetic acid was ever detected. It seemed possible if not probable, that the effect of hyperventilation was, in this case, compensated so that alkalosis did not develop. The blood studies were made to determine the nature of the compensatory mechanism.

At the time of the first examination total base and total acid were both within normal limits. Bicarbonate was extremely low, only 21.2 volumes per cent of total  $\text{CO}_2$ . Protein was somewhat high, phosphate and undetermined acids were normal. Chloride was considerably elevated and had evidently replaced the bicarbonate deficiency. pH was not determined, but can be estimated very roughly from the respiratory study.

If it is assumed that the dead space is 150 cc. and that the expiratory air beyond this comes from the alveoli and is approximately at the same tension as the arterial blood, one can calculate the arterial  $\text{CO}_2$  tension from the  $\text{CO}_2$  of the expired air and the effective minute volume, by the following formula:

$$\frac{100 \text{ CO}_2}{V - 150 R} = A \quad (2)$$

in which  $\text{CO}_2$  represents carbon dioxide consumed per minute;  $V$  the total volume of air expired per minute;  $R$  the number of respirations per minute; and  $A$  the per cent of  $\text{CO}_2$  in the alveolar air.  $A (B - w) =$  alveolar or arterial  $\text{CO}_2$  tension, where  $B$  is the barometer reading under standard conditions and  $w$  the water vapor tension at body temperature.

The arterial  $\text{CO}_2$  tension calculated by these equations from the first respiratory run, in this case would be

$$\frac{100 \times 1.85}{18,500 - (30.4 \times 150)} (764.5 - 49.4) = 17.5 \text{ mm.}$$

The pH of the serum, using the Henderson-Hasselbalch equation with a  $\text{pK}_1$  of 6.10, would be 7.32. The validity of these calculations is open to serious question in this experiment not only because of the uncertainty of the dead space volume, but also because the arterial puncture and the respiratory run were not made simultaneously. The values obtained do, however, probably give an idea of the order of magnitude of the arterial tension, even though they have no absolute value. If this is granted it is obvious that hyperventilation in this case did not result in alkalosis, but was compensated by a reduction of bicarbonate and a proportionate rise of chloride.

At the time of the second experiment, as has been already pointed out, the condition of the patient had improved considerably, and during the run there was no evidence of over-

ventilation. Examination of the blood this time revealed an increase of  $\text{CO}_2$  to almost twice its original value. Total base and total acid had both increased somewhat; protein and chloride had fallen. This time pH was determined gasometrically by the technique described above and equation 1.

In the experiment under discussion the separated serum, saturated at 20 mm., contained 38.8 volumes per cent of  $\text{CO}_2$ , and at 60 mm., contained 48.3 volumes per cent of  $\text{CO}_2$ . The  $\text{CO}_2$  tension of the blood in the body was, therefore, 30.3 mm. and the pH 7.37, an entirely normal figure. Calculated from the respiratory study the alveolar  $\text{CO}_2$  tension proved, from equation 2, to be 30 mm. This illustrates the value of this method of approximating the  $\text{CO}_2$  tension when respiration is normal. It has been employed by Krogh and Lindhard, Pearce, and by one of the authors in another connection (5).

In the third experiment the administration of bicarbonate had resulted in elevating the serum  $\text{CO}_2$  somewhat further, again at the expense of the chlorides. The pH was again determined gasometrically. In this case serum  $\text{CO}_2$  at 30 mm. was 43.3 volumes per cent, and at 60 mm. 51.7 volumes per cent. The  $\text{CO}_2$  tension of the blood as drawn, calculated from these points by means of equation 1, was 32.8 mm., giving a pH of 7.38. From the respiratory study the  $\text{CO}_2$  tension by equation 2 came out 28.8 mm.

For the disagreement in the last case between the arterial  $\text{CO}_2$  tension calculated by the two methods a partial explanation may be found in the irregularity of the breathing during the experiment. The spirometer gas on which the calculations were based was the product of the whole 10 minutes, while the blood was obtained during 3 of these minutes only. The effective ventilation was not, however, especially small during these minutes. It is more probable that during hyperventilation, especially when the respiratory rate is unduly increased, the air is less effectively mixed than usual. This would have the same effect as an enlargement of the dead space of the lungs and would invalidate calculations of the alveolar  $\text{CO}_2$  from expired air by a formula that involved the use of an assumed dead space value.

It is clear from the gasometric pH determinations that the pH of the blood during the second two experiments was normal.

About the first there must be more uncertainty. It is, however, highly unlikely that the patient had any alkalosis at this time. The errors in calculations derived from the expired air would all tend to minimize the  $\text{CO}_2$  tension and, therefore, to exaggerate the pH. Furthermore, with the observed serum  $\text{CO}_2$  content the arterial tension would have had to be as low as 14 mm. to give a pH of 7.4 and at the extreme low level of 11.4 mm. to raise the pH to 7.5.

It is reasonably certain that in this case definite overventilation occurred at frequent intervals, sometimes persisting for long periods, without the appearance of tetany and it seems to be more than a simple coincidence that the overventilation did not produce alkalosis. The overventilation did, however, result in a reduction of the  $\text{CO}_2$  tension. There was, at the same time, a replacement of bicarbonate by chloride, which compensated for the reduction of  $\text{CO}_2$  and was primarily effective in preventing alkalosis. Whether any significance is attached to the alterations of total base one cannot say. The base did, however, vary in the same direction as bicarbonate and reduction of base may have played its part in preventing the development of alkalosis.

It is impossible to say from the blood studies alone whether high chloride or low bicarbonate represents the primary disturbance; but the clinical condition favors the latter interpretation of the data. The disease from which the patient suffered affected the central nervous system. Apparently the respiratory mechanism had escaped from the normal control and responded wildly to extraneous stimuli. The heart and the vasomotor system showed a similar tendency. Apparently the response to intrinsic stimuli was comparatively normal because, when he was asleep, pulse and respirations lost their unusual character. Evidence of such a dissociation is also seen in the first experiment. During the second run, when he went to sleep, the ventilation quieted down and, indeed, frequent periods of apnea developed. Even under these conditions, however, there was an abruptness and violence in the periodic respiration that is not usually observed in normal subjects after overventilation.

If it is assumed that overventilation was the primary cause of the changes in the blood electrolytes, it follows that the chloride response must have taken place with extraordinary rapidity.



*If the bicarbonate reduction were effected by means of the respiratory mechanism alone, alkalosis could hardly have failed to ensue unless chlorides increased almost as rapidly as CO<sub>2</sub> diminished. At the time of the third experiment a freshly voided specimen of urine was examined and found to be distinctly alkaline (it contained no acetone). Apparently the kidneys played their part in compensation, probably by excreting bicarbonate, even though the pH of the serum was not high. The administration of extra bicarbonate placed an additional load on the mechanism, but did not succeed in overcoming the defenses of the organism and producing alkalosis.*

In any event it is obvious that the organism can and does mobilize chloride for the maintenance of neutrality in response to changes of bicarbonate. The response to overventilation in this case is quite different from that of JP. This may be due to the difference in duration and intensity of the excessive breathing. From measurements in unpublished experiments it has been found that the effective minute volume of JP during voluntary hyperventilation far exceeds that of the patient under discussion.

The first reaction to extremely rapid and short hyperventilation may consist in a reduction of free CO<sub>2</sub> and chlorides. If the hyperventilation could have been prolonged at this excessive rate it is possible that chloride would have increased. As it was, organic acid increased, perhaps as a result of tetany. When overventilation is carried on more slowly for a long period, chloride may respond so completely that no alkalosis develops and organic acid does not appear. The resulting picture, when judged from the standpoint of the blood alone, is indistinguishable from that of a compensated chloride acidosis. There may be a slight recession of total base to aid in the adjustment. The end-result is conservative in preventing disturbances in the reaction of the blood which would inevitably result in frequent or continuous tetanic convulsions.

#### *Vomiting and Gastric Tetany, a Study of Two Cases.*

About the same time there was admitted to the medical service a patient with vomiting, mild hyperventilation, and fully developed tetanic symptoms.

*Case 18406.*—American, female, age 43, admitted to the hospital June 2, 1925. 2 years earlier her first pregnancy had been terminated during the 8th month because of a toxemia. At that time she was vomiting and complained of pain in the back and the epigastrium, frequent severe headaches, loss of vision, and slight swelling of the lower extremities. Her systolic pressure was over 200, diastolic about 140; she had a well developed optic neuritis and retinitis, slight puffiness of the face and lower extremities, and moderate exophthalmos. The urine contained considerable albumin and occasional granular casts. The blood non-protein nitrogen was 26 mg. per cent; phenolsulfonephthalein excretion 30 per cent in 2 hours. Blood Wassermann was negative. She gave a history of dyspnea on exertion antedating the onset of pregnancy. The child died immediately after delivery, but the patient improved rapidly. The symptoms ceased, the systolic blood pressure gradually fell to 150 and the diastolic to 100; the optic neuritis receded; and the phenolsulfonephthalein excretion rose to 60 per cent. The blood non-protein nitrogen at the time she left the hospital was 29 mg. per cent.

The patient returned from time to time after this for observation. On each occasion she complained of occasional headaches, nausea, and slight swelling of the feet. The blood pressure gradually rose again to its original high level; the systolic pressure was 180 mm. in July, 1923; 240 in March, 1924; 248 in June; 250 in July; and 240 in September. The diastolic pressure at the last observation was 140 mm. At this time, September, 1924, she again complained of blurring of vision. In the early part of May, 1925, the headaches became more severe, she developed morning sickness, vomiting frequently, and noticed that her urine was becoming scanty. The vomiting gradually increased in frequency and severity until for a few days before she entered the hospital she was unable to retain any food at all. During this period she became somewhat drowsy. At times she had attacks of breathlessness during which she developed spasms of her hands and feet. The night before admission she had a more severe attack than usual in which the spasms became so marked that she was unable to move.

When brought into the hospital she had another of these attacks in which she was seen by the members of the staff. The picture presented was quite typical of tetany with striking carpopedal spasm and a positive Chvostek's sign. On examination she appeared flushed and, at times, somewhat cyanotic. Her breathing was irregular. Sometimes she pumped rather violently; at other times the respirations subsided and became quite slow and shallow. These changes were evidently referable in part, at least, to psychic factors because the overventilation occurred especially when she was disturbed or examined. During the periods of hyperventilation she usually developed tetanic spasms of the extremities. The eyes were protuberant, with all the signs of exophthalmos. There was striking edema of both optic nerve heads. The systolic blood pressure was 230; diastolic 135. The heart was somewhat enlarged. There was no edema of the extremities. She appeared dehydrated and complained of thirst, but was too nauseated to take anything by mouth. The urine contained a trace of albumin and

a few hyaline casts. Blood count 4.7 million red blood cells; 11,500 leucocytes with 78 per cent of polymorphonuclear neutrophils. The blood non-protein nitrogen was 36 mg. per cent.

Blood was withdrawn from an artery shortly after the patient entered the hospital. A little before and a little after the arterial puncture she had attacks of overventilation and tetany, but while the blood was being withdrawn the respirations were little increased and she had no spasms. She was immediately given a hypodermoclysis of 1500 cc. of normal saline solution. She was ordered carbohydrate fluids and salt by mouth, but little was given during the first 24 hours because of the persistent nausea and severe headache. The hyperventilation and tetanic seizures ceased, however, soon after the administration of the saline and by the next morning the headache and nausea had also disappeared. Owing to an error salt was not added to the diet for the first few days. After the 2nd day she was given a high carbohydrate diet with 50 gm. of protein, and on June 9 this was increased to 60 gm. The basal metabolism was determined June 5 and proved to be quite normal. The expiratory minute volume at this time was 5000 cc., the respiratory rate 21.7 per minute, and the expired air contained 2.87 per cent of  $\text{CO}_2$ . The second blood examination was made the morning of June 9, before breakfast. She was then entirely free from symptoms of all kinds. She was discharged June 13. Her systolic blood pressure was 190, diastolic 130. The results of metabolism studies and blood examinations appear in Tables IX and X. *Impression:* Essential hypertension (malignant); vomiting; overventilation tetany.

The first blood contained the normal amount of bicarbonate, but less than the usual amount of chloride. The total base was also somewhat low. After recovery almost every element was altered.

In the first place the serum proteins had diminished. This would seem to indicate that the blood had taken up water. However, oxygen capacity, which should also be affected by such a change, remained unaltered. The reason for this discrepancy is found in the cell volume values. Apparently the cells shrunk during the improvement. If hemoglobin be considered a measure of the water content of whole blood, one can say that the hydration of the blood has not changed. The relative volume of the plasma in the second observation is, however, greater than that of the first as  $100 - 35.9 : 100 - 40.6$  or  $64.1 : 59.4$ . These figures are almost the exact reciprocals of the serum protein values 5.96 and 6.35. The agreement is almost too good to be the result of coincidence. It seems more likely that the serum has really gained water at the expense of the cells. Be this as it may, it is

TABLE IX.  
*Total Acid-Base Equilibrium of the Plasma of Three Patients with Tetany.*

Case No.	Date.	Oxygen capacity.	Cell volume.	Plasma.							Blood non-protein nitrogen.	Nature and treatment of blood.	Remarks.	
				Protein.	CO <sub>2</sub>	Cl	Inorganic P.	Total acid.	Total base.	Organic acid.				
		vola. per cent	vola. per cent	per cent	vola. per cent	mg. per 100 cc.	mg. per 100 cc.	mm	mm	mm	mg. per 100 cc.	(12)	(13)	
18406	June 2, 1925	15.3	40.6	6.35 s	60.9	337	2.7	133.3	145.1	11.8	36	A. cont.		Malignant hypertension. Tetany. Vomiting. Overventilation.
22459	" 9, "	15.3	35.9	5.96 s	61.4	352	3.5	137.4	158.1	20.7	23	"	"	After recovery from symptoms.
	Aug. 13, 1923				53.1	313		124.7				V.	"	Typhoid fever. Vomiting. Overventilation. Tetany.
35417	Dec. 15, 1924			7.09	64.0		3.8		200.6			"	"	Psychoneurosis. Hysteria. Tetany.

TABLE X.  
General Metabolism Data of Subject 18406.

Date.	Fluid intake.	Urine.	Fluid balance.	Cl			Food.		Urine nitro-gen.	Nitro-gen balance.	Blood non-protein nitro-gen.	Remarks.
				In-take.	Urine.	Balance	Calo-ries.	Nitro-gen.				
	cc.	cc.	cc.	gm.	gm.	gm.		gm.	gm.	gm.	mg. per 100 cc.	
1925 June												
2	2,670	610	2,060	8.0	1.4	6.6	300	0	6.1	-6.1	36	1500 cc. saline hypodermoclysis and orange juice by mouth.
3	1,500	920	580	0.0	1.4	-1.4	310	1.9	4.8	-2.9		
4	2,250	950	1,300	1.2	0.6	0.6	1,020	7.5	4.2	3.3		
5	3,200	1,460	1,740	1.4	1.1	0.3	1,720	10.2	9.6	0.6		
6	3,800	1,270	2,530	3.2	1.0	2.2	1,020	7.5	8.6	-1.1		
7	3,600	1,370	2,230	7.5	3.2	4.3	1,720	10.2	7.3	2.9		
8	3,800	2,690	1,110		19.9		2,560	9.2	10.1	-0.9		Allowed unlimited salt in diet and given additional 10 gm. in capsule form.
9		1,700			14.0		2,030	9.2	6.8	2.4	26	
10		2,220			14.8			9.2	9.5	-0.3		
11		2,045			9.9		2,400	9.4	7.7	1.7		
12		1,570			6.2			9.4	7.0	2.4		

at least certain that the cells at the time of the first observation contained more water for each unit of hemoglobin than they did after recovery. Furthermore, the relative water content of the cells was high in relation to normal standards. According to the method employed in this work the average value of the ratio, *oxygen capacity : cell volume* in defibrinated blood of normal individuals is about 0.42.<sup>3</sup> In the first blood specimen in this case it was only 0.38; in the second it had returned to normal, 0.43. For this peculiar tendency of the cells to become relatively or absolutely excessively hydrated in conditions in which the organism as a whole appears to be dehydrated no explanation offers itself. It has, however, been frequently encountered by us and will be referred to in other connections. Gamble and Ross (11) have recently shown that the serum protein concentration increases after pyloric obstruction and interpret this as an indication that the serum has lost water. As long as discussion is limited to serum such a conclusion is probably warranted, but it cannot be assumed from the same data that the blood as a whole has become dehydrated.

Gamble and Ross (11) in the same paper have pointed out that pyloric obstruction leads to a depletion of the chloride and, to a lesser degree, of the sodium of the plasma. Our findings are quite in keeping with theirs. The deficiency, according to them, may be ascribed to an excessive loss of these elements in the vomitus. Haden and Orr (13), on the other hand, do not believe that the loss of chloride in the vomitus plays an important part in determining the low level of blood chloride. Although the case under discussion is not one of pyloric obstruction, the vomiting had been so persistent that the condition is more or less comparable to that produced by pyloric obstruction. The changes in the serum are also quite typical except for the fact that bicarbonate is normal. The patient, unfortunately for the investigators, ceased to vomit almost as soon as she entered the ward, so that the chloride and base lost in the vomitus could not be estimated. The scanty metabolism data given in Table X, however, show that she retained both fluid and salt in large quantities during recovery. This may be considered as presumptive evidence that she had

<sup>3</sup> In oxalated blood it is about 0.46.

suffered an excessive loss of the same elements during the preceding period.

If the defense of the body against the alkalosis of overventilation lies in the replacement of carbonic acid by chloride, the cause of tetany in this case is not hard to find. With chlorides depleted by vomiting compensation could not be effected. In consequence overventilation of a grade that reduced bicarbonate only to the normal level resulted in the development of true tetanic spasms. It is unfortunate that pH was not determined. It is more than probable that it would have been found to be abnormally high.

The blood non-protein nitrogen is usually found to be elevated in cases of organic obstruction of the gastrointestinal tract (23, 7, 14). This elevation has been ascribed by Haden and Orr (14) and others to a toxic destruction of protein. In this case the non-protein nitrogen was not abnormally high, but it was lower at the time of the second blood examination. Study of the nitrogen balance reveals no evidence of abnormal nitrogen catabolism. The excretion of nitrogen did increase strikingly, however, as soon as diuresis was established.

The low phosphate encountered in the first studies of this and the preceding patient are interesting in relation to the results of the anoxemia experiments. It may be that reduction of serum phosphate regularly follows overventilation.

Case 22459 presents a similar condition. In this instance vomiting occurred as a complication of typhoid fever. The chloride reduction is even more marked than that of the preceding case and bicarbonate is also lower. With the chloride defense so seriously compromised it is easy to understand why alkalosis and tetany developed under the influence of moderate overventilation and without any elevation of bicarbonate.

*Case 22459.*—Danish, female, age 31, admitted to the hospital August 8, 1923, in the 2nd week of typhoid fever, which had occurred in the 2nd month of pregnancy and precipitated a miscarriage. On August 11 she developed a mild diarrhea and at the same time began to vomit frequently. 2 days later carpopedal spasm was observed. For 24 hours previous to this her respirations had been rapid, at times rising above 40. It was during these periods of dyspnea that signs of tetany appeared. There seemed to be a distinct psychic factor involved, because the dyspnea increased when she was examined or knew that she was observed. The blood was examined on

August 13. At this time the temperature was about 103°F., pulse 110, respirations about 30 per minute. Vomiting ceased the next day, but she proved difficult to feed, the diarrhea persisted, and tetanic symptoms recurred at intervals for some days longer. After a protracted and complicated illness she was discharged from the hospital, cured, in the middle of September. The results of the blood examination appear in Table IX.

*Case 35417.*—American, female, age 32, married, admitted to the hospital, December 13, 1924. For 3 years she had had attacks of needle-like pains, beginning in the left hand, gradually travelling up the arm and spreading to the trunk and the other limbs. These gradually increased in severity. With the pains came a sense of numbness. In August, 1924, she had a seizure of muscular rigidity that suggested tetany, during which she became unconscious. She had two milder attacks subsequently. She appeared well developed and nourished. Temperature was normal; the pulse 80 to the minute with a rather striking sinus arrhythmia; the systolic blood pressure was 104, diastolic 75. The palms of her hands were dripping with perspiration and her pupils were abnormally dilated. Otherwise the physical examination and special examinations including blood Wassermann test, urinalysis, blood count, gastrointestinal x-rays, x-ray examination of the skull, gastric test meal, and basal metabolism, proved negative. Blood was examined before breakfast on December 15. She had no symptoms at the time. *Impression:* Tetany.

The results of the blood examination appear in Table IX.

Case 35417 presents an entirely different blood picture associated with definite tetanic symptoms. The patient was admitted to the private pavilion under the care of an outside physician and it was impossible to make a complete study or to repeat the examination of the blood. The striking thing in this case is the high level of total base. Bicarbonate is high, but does not exceed the extreme normal limits. In this instance it seems likely that the excessive amount of base in the serum rendered the patient more susceptible to alkalosis and tetany. Serum calcium was quite normal.

#### DISCUSSION.

In certain respects these experiments throw some light on the probable nature of the differences between arterial and venous blood shown in Paper III (22). When the venous blood from a local part of the body is altered under the influence of extraneous conditions, this alteration can only come about if the arterial blood is changed as it passes through the part. Although arterial blood was not taken, the preliminary venous blood, es-



pecially after the arm had been immersed in hot water, was more nearly like normal arterial blood than was the second specimen, taken after stasis or exercise, and the difference between the two must be qualitatively similar to the difference between arterial and venous blood. Studying the blood before and after a given disturbance is equivalent to magnifying the change that occurs between arterial and venous blood during this disturbance, because no alteration of the constitution of the blood can take place except by abstraction or addition of material in the tissues.

TABLE XI.

*Changes in the Carbon Dioxide Absorption Curves of Whole Blood and Plasma as a Result of Venous Stasis, Exercise, and Overventilation.*

Sub- ject.	Date.	Vols. per cent CO <sub>2</sub> in:						Remarks.
		Blood at CO <sub>2</sub> tension of:			Plasma at CO <sub>2</sub> tension of:			
		30 mm.	40 mm.	60 mm.	30 mm.	40 mm.	60 mm.	
JP	1924							
	Mar. 18	42 2		53 4	49.9		63 5	Before stasis.
		34 8		49.7	43 5		61 2	During “
	“ 27	39.5		51 9	46.2		60.0	Before exercise.
		34 9		47.6	40.3		54 5	During “
	Apr. 16		44.7			53 0		Before overventilation.
		42.9			52.6		During “ tetany.	

The types of change that can occur are quite various and depend, as we suggested in the preceding paper (22), on the nature of the provoking disturbance. In the stasis experiment the water and chlorides of the blood changed; in tetany chlorides fell; in exercise base rose. Each one of these changes affected the carbon dioxide absorption curve of both blood and serum in a different manner. The nature of these effects is shown in Table XI. One cannot assume that because any one of the constituents of blood is unchanged the reaction of the blood to carbon dioxide will remain unaltered.

The rapidity with which chloride and base respond to disturbances in the other electrolytes is striking. In this response chloride must play a part quite analogous to that which it plays in the transfer of electrolytes across the cell membrane. Any

diminution of chlorides without a corresponding loss of base liberates base to combine with carbonic or other acids and, therefore, tends to prevent excessive acidity and to promote a state of alkalosis. *Vice versa* the passage of Cl from the tissues to the blood has the opposite effect. Presumably the Cl is released or taken up by base in the tissues and this base, like that of the blood cells is, therefore, rendered available for the maintenance of the reaction of the plasma and the general exchange of electrolytes throughout the body.

The objection may be raised that the data thus far presented do not prove that Cl actually passed into the tissues and not

TABLE XII.

*Changes in the Chlorides of the Blood and Their Distribution under Various Conditions.*

Subject.	Date.	Blood.		Plasma.		Cells.	
		Concentration.	Actual amount.*	Concentration.	Actual amount.*	Concentration.	Actual amount.*
	1924	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm
JP	Mar. 11	274	77.3	370	59.8	146	17.5
		260	60.7	349	39.6	169	21.1
	Apr. 3	292	82.2	359	62.6	183	19.6
		291	80.1	359	58.0	195	22.1
	" 10	291	82.2	368	60.1	188	22.1
		283	81.1	355	59.8	180	21.3
	" 16	290	81.6	374	59.1	181	22.5
		283	75.6	364	54.6	178	21.0

\* Millimols of Cl in the plasma, cells, or whole of a liter of blood.

merely into the blood cells. The answer to this objection is given in Table XII which shows the whole blood Cl before and after stasis, exercise, and tetany. Column 1 gives the chloride concentration observed, Column 2 the actual amount of chloride per unit of original blood. The succeeding 4 columns give similar data for plasma and cell chlorides. The methods of calculation are similar to those applied to the analysis of arterial and venous blood in the preceding paper of this series (22). The direction of the changes in concentration and total amount of chloride in the blood reflect the similar changes encountered in the plasma. Although the two differ in magnitude they are always the same in direction.

*In the past observers, confining their studies largely to carbon dioxide and oxygen changes and the hydrogen ion concentration, have obtained an unbalanced view of the purpose and effect of the acid-base mechanism. A certain group has insisted that the hydrogen ion concentration of the blood played the dominant rôle in determining the automatism of the respiratory mechanism, others have ascribed the same function to carbonic acid. Neither group has been able to maintain its theory in the face of the growing body of experimental data. In oxygen-want, for example, dyspnea is found associated with a reduced bicarbonate and an elevated pH (3). The alkalosis that follows the administration of bicarbonate is not attended by a cessation of respiration. Even the most ardent exponents of the theory that pH regulates respiration have been forced to admit that this chemical control is frequently replaced by some other stimulus.*

Y. Henderson (15) in a reaction against the restricted viewpoint of the chemists has called attention to the fact that the true function of respiration is to supply the tissues with adequate oxygen and to relieve them of sufficient carbon dioxide to carry on their normal functions and has suggested that the maintenance of a constant blood pH may be only an incidental result. From this point of view he argues that respiration is dependent on a combination of two factors, the alkali and the oxygen tension of the blood. The argument is most ingeniously developed and seems to explain many of the phenomena of respiration for which the pH theory failed to account, and especially the dyspnea of oxygen-want. The chief criticism against Y. Henderson's hypothesis depends on the fact that he used the terms alkali and bicarbonate as if they were synonymous. To be sure, he did qualify this by adding the adjective "available" in some connections; but from his discussion it is not at all clear that he appreciated the restrictive significance of the term "available alkali." This term, like the term "alkaline reserve," is of doubtful value. At best it applies only to the discussion of test-tube experiments. It is quite evident that all the alkali in the cells and in the plasma is available for the neutralization of acid and that the tissues contain a further store which forms a highly mobile "alkaline reserve."

Y. Henderson discredits the idea that the acidosis of diabetes

and the symptoms that ensue are due to the abstraction of base from bicarbonate by the ketone acids. The latter, he believes, enter the blood as neutral salts, already combined with base. At this point he is not entirely clear, but gives the impression that the blood base increases without any direct association with bicarbonate changes.

A highly mobile, fluctuating blood alkali such as Henderson postulates would result in frequent and extreme variations in the osmotic pressure of the serum. The latter must be in large part determined by the concentration of electrolytes. This is defined by the sum of the cations and anions. As blood is practically neutral and all the important organic elements, especially the protein, behave as anions, total base may be used as a measure of the fraction of the total osmotic pressure which is contributed by electrolytes.

All determinations of the osmotic pressure of serum have shown that it is comparatively constant even under the most diverse conditions of health and disease. Gamble and his associates (10) have, furthermore, shown that the total base of serum is maintained at a very uniform level by the action of the kidneys and the tissues. They have also shown that chloride can be made to replace bicarbonate. They have not, however, attempted to demonstrate the reverse of this process. In consequence they have concluded that bicarbonate holds a "mendicant position," giving way to every other acid that enters the blood. Bicarbonate is no more mendicant than chloride, and may remain constant or increase while the latter diminishes. It has been demonstrated that chloride may be forced to replace bicarbonate when the latter is depleted. Finally, the exercise experiment showed that base itself may be compelled to depart from its fixed and even course under the proper stimulus.

Current theories that postulate a greater constancy for one element or function of the blood than for another cannot survive the results of such experiments as these, and there is no good evidence that any element is especially favored at the expense of another. Undoubtedly any alteration of equilibrium relations will invoke a compensatory reaction that will tend to minimize the effect of that disturbance. This is true in any chemical or physical system. In a system as complex as that of the blood,

especially when it is in the body in contact with the still more complex tissues, alteration of even a single component must initiate a train of reactions that affects not another single component, but almost every constituent of the blood, and the influence of which will extend far into the tissues.

In the rearrangement and adjustment which ensues each one of these components will yield somewhat and each will resist until an equilibrium is again restored under conditions as near as possible to the normal state. A short or trivial disturbance must necessarily initiate one set of reactions; a more prolonged or drastic disturbance will elicit a different response. The effect of any condition will depend on the nature, intensity, and duration of the disturbance it provokes; but this effect will seem to have a conservative tendency.

There is no reason for confining the term equilibrium to electrolytes nor the term components to recognizable individual constituents of the blood or tissues. The processes of metabolism that occur in the tissues are almost certainly reactions in a state of equilibrium and the process of respiration itself must be included as a component of the system. In this respect a biological system differs from a test-tube experiment. If such a conception is admitted it is impossible to entertain the idea that respiration can respond to pH only, without reference to the metabolic needs of the body, or that osmotic pressure will yield to no other function of the blood. The disturbance of metabolism that would result from such an unbalanced reaction would, in itself, inevitably initiate further reactions that would again have a conservative effect. Biological automatism is an undeniable fact. It is quite as obvious that there must be an interaction between all the various parts of the whole automatic organism. One can, then, consider the reactions that occur in response to a given disturbance as tending towards the maintenance of the normal bodily functions as a whole without accepting a vitalistic philosophy.

In the stasis experiments the most striking phenomenon is the loss of water from the blood to the tissues. Estimations based on the cell volume and oxygen capacity figures alone in the experiments of March 11 and April 18 show that this loss was borne almost entirely by the plasma and amounted to 30 per cent of the

volume of the plasma. The reaction is quite like the loss of water to the blood cells after the addition of  $\text{CO}_2$ ; but in this case the blood cells are left unchanged and the tissues gain all the fluid. Furthermore the reaction is not simply a response to  $\text{CO}_2$  because it does not occur in either of the exercise studies or after combined exercise and stasis, although both  $\text{CO}_2$  and pH are quite as much or more altered in these experiments. The occurrence of serum dehydration in oxygen-want experiments leads one to wonder whether anoxemia may not also be the factor that determines the transfer of water in stasis. This loss of water may represent an effort to dilute the waste products of metabolism which have accumulated in the tissues because they are deprived of their normal means of egress from the body. The metabolism may itself be compromised and rendered abnormal by lack of oxygen and the accumulation of these products. The transfer is probably a result of increased capillary pressure possibly aided by elevation of the osmotic pressure in the tissues due to the accumulation of catabolites. One result of the fluid exchange may be to permit a larger proportion of the carbon dioxide load to be carried by the more highly buffered tissue cells.

Concerning the value of the diminution of chloride there can be little doubt. In the first place by rendering its base available for combination with these acids it lessens the pH alteration that would occur if bicarbonate alone carried the  $\text{CO}_2$  and acid load. Furthermore the blood is enabled to carry more  $\text{CO}_2$  at a lower tension. The latter point must be of prime importance in permitting metabolism to continue in a comparatively normal manner. With all opportunities for carbon dioxide to escape cut off, no set of reactions could well be conceived to meet the situation with less ultimate disturbance of the original equilibrium conditions and the metabolic processes of the tissues.

In exercise the blood water was not seriously altered and the distribution of fluid between the two phases of the blood was not affected in any characteristic manner. The main problems to be met in exercise are, first the elimination of the excessive amount of carbon dioxide produced by the heightened metabolism, and second the neutralization of lactic acid. The provision of an adequate oxygen supply is secondary during short periods of exercise, at least. Neglecting for a moment the pulmonary

*ventilation, which is probably not seriously affected by such limited local exercise as that of the present experiments, the delivery of carbon dioxide from the blood will be facilitated as the CO<sub>2</sub> tension of the blood increases. By sparing chloride and forcing bicarbonate to bear a larger part of the burden the CO<sub>2</sub> tension is elevated and the pH falls. This fall is not, however, great enough to carry the whole of the added acid. The remainder is sustained by the abstraction of base from the tissues and not from chloride.*

When tetany is rapidly induced by overventilation bicarbonate gives up little of its base, although by doing so the change of reaction that is generally considered to be the cause of the tetanic symptoms would be diminished or prevented. Foreign organic acids which should aid in promoting such compensation do appear, but are entirely taken up by base provided by chloride. It is worthy of note that in overventilation the removal of CO<sub>2</sub> far exceeds the demands of metabolism. If base were ceded to Cl by bicarbonate the reduction of CO<sub>2</sub> tension and elevation of pH would be minimized. The very fact that the CO<sub>2</sub> tension remained relatively high would result in the loss of an excessive amount of CO<sub>2</sub>. Direct loss of base to the tissues would have the same effect and would, furthermore, lower the osmotic pressure of the blood.

The effect of oxygen-want on the blood electrolytes is quite as variable as is its effect on respiration and the results of the three experiments of this series indicate that there is some relation between the two sets of phenomena. This is further borne out by the study of Case 28976, with postencephalitic hyperventilation. Apparently, if hyperpnea of moderate degree due to causes other than excessive CO<sub>2</sub> production continues for a long enough period, Cl is yielded by the tissues to replace the base freed by bicarbonate, thus preventing the development of serious alkalosis and forestalling tetany. Just why a similar reaction should not occur after short, violent overventilation is not clear. Time alone cannot be the determining factor. Chloride can respond in the reverse direction, at least, with surprising rapidity, as the stasis and voluntary hyperpnea experiments indicate. Some may object that the reduction of chlorides in response to hyperpnea is not a general reaction but one peculiar to the subject JP.

This is possible, but hardly probable. Furthermore it is the same subject who presented the reverse change in response to the hyperpnea of oxygen-want. At any rate it has been demonstrated by these observations that the organism is capable of compensating for reductions of bicarbonate by substituting chloride, and that this reduction diminishes the pH change that would otherwise result. No generalizations about the effect of low oxygen tensions are warranted on the basis of these observations. The more gradual or prolonged production of anoxemia may initiate an entirely different set of compensatory reactions. On the other hand this work compels reconsideration of the theory advanced by Y. Henderson and now rather generally accepted, that the reduction of bicarbonate at high altitudes is followed by withdrawal of base from the blood. As far as we know the level of serum chloride at high altitudes has not yet been investigated. On theoretical grounds an increase of chloride is more probable than a reduction of total base, because the latter would entail an alteration of total electrolyte concentration and osmotic pressure. Koehler and his associates (16) have recently shown that, although the primary reaction to low oxygen pressure is the production of an overventilation alkalosis, acidosis supervenes if oxygen deprivation is carried to an extreme degree or greatly prolonged. The degree of oxygen reduction required to induce such an acidosis varied greatly in different animals. The experiments on JP, June 18, and HAB, June 26 (see Table IV), perhaps offer a clue to these variations in reaction. The alkalosis may well be simply the response to hyperpnea and not directly related to oxygen-want. The acidosis, which develops only in extreme stages may be the direct result of anoxemia. Koehler *et al.* (16) recognize the probable importance of organic acids in the production of this acidosis and further suggest that phosphates play a part. The latter suggestion is not borne out by our experiments in which serum phosphate fell.

The fact that both in exercise and in oxygen want the added load of organic acid was borne by base derived from the tissues leads one to wonder whether this does not represent the usual reaction to the rapid accession of organic acid.

The last cases show that severe vomiting, due to conditions other than obstruction of the gastrointestinal tract, may reduce



the chlorides and base of serum and that under these conditions, when the chloride defense is weakened, even moderate hyperpnea quickly leads to tetany.

#### SUMMARY AND CONCLUSIONS.

Prolonged venous obstruction leads to the transfer of water from the blood to the tissues and a concentration of the proteins. Base combined with bicarbonate is unavailable for the neutralization of this excess acid because the usual escape of  $\text{CO}_2$  through the lungs is prevented by the presence of the tourniquet. Under these circumstances plasma chloride diminishes, yielding its base to protein and carbonic acid.

In brief, vigorous exercise considerable lactic acid and an excess of carbonic acid are formed, and the pH of the serum falls. Chloride remains unchanged. Bicarbonate cedes some base to the organic acid, but the major portion of the latter is neutralized by base yielded from the tissues.

If overventilation is produced as rapidly as possible, symptoms of tetany appear when the pH has risen by not more than 0.2. Although the total  $\text{CO}_2$  of the serum falls, the carbon dioxide capacity remains unaltered. Organic acid, probably partly ketone acids, but mostly lactic acid, is considerably increased. The total base remains unchanged and the base required for the neutralization of the foreign acids is largely derived from the chlorides, which are diminished.

The reaction of the electrolytes to oxygen-want varies according to the respiratory response. If moderate overventilation develops and continues for a long time bicarbonate falls. The  $\text{HCO}_3$  is replaced partly by an increase in the concentration of serum protein, but chiefly by Cl withdrawn from the tissues. If extreme anoxemia is produced organic acid rises and is neutralized by base derived from the tissues.

A postencephalitic case presenting overventilation without tetanic symptoms compensated for an extreme reduction of  $\text{CO}_2$  by an equivalent increase of chloride. On three occasions chloride and bicarbonate were found to have changed, always in reciprocal directions.

A malignant hypertension case and one with typhoid fever by vomiting depleted the chlorides of the serum. Under these cir-

circumstances tetany followed mild overventilation. The loss of Cl appears to diminish the ability of the organism to prevent tetany by readjusting the acid-base balance after loss of  $\text{H}_2\text{CO}_3$ .

It is suggested that all these reactions are conservative in nature. Any given disturbance of electrolyte equilibrium will evoke a train of reactions and changes in all the other electrolytes. These reactions always tend to restore equilibrium. The response is not directed towards the maintenance of the concentration of any single constituent or group of constituents, but will be manifested to a greater or less degree in one or the other according as it may best serve to restore the equilibrium and maintain the functional automatism of the whole organism.

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## TOTAL ACID-BASE EQUILIBRIUM OF PLASMA IN HEALTH AND DISEASE.

### V. MISCELLANEOUS PATHOLOGIC CONDITIONS.

BY JOHN P. PETERS, HAROLD A. BULGER, ANNA J. EISENMAN,  
AND CARTER LEE.

*(From the Department of Internal Medicine of Yale University and the  
Medical Service of the New Haven Hospital, New Haven.)*

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#### *Effect of Vomiting.*

The acid-base equilibrium of a group of patients with miscellaneous pathologic symptoms was determined by the methods outlined in Paper I of this series (4). The results of these studies are presented in Table I and protocols of the cases are given for reference at the end of this paper. The cases were largely selected because they presented conditions that might be expected to disturb the electrolyte equilibrium of the blood or disorders analogous to those encountered in diabetes and nephritis.

The first four patients were all admitted because of severe vomiting due to one cause or another. The first, Case 34622, had taken phenol and had vomited continuously for about 12 hours. The only disturbance of any significance was the presence of a rather low base. Chloride had not been appreciably reduced. Vomiting did not persist long enough to produce ketosis. In the next case severe vomiting had persisted for some years. Nevertheless blood findings were quite normal. It is reasonably certain that the patient retained a considerable amount of food in spite of the emesis; otherwise she could hardly have maintained her weight and strength as she did nor could she have avoided ketosis. This and the fact that the vomitus was deficient in acid may explain the fact that the electrolyte equilibrium of the blood was unchanged.

Patients 26515 and 35556, both with toxemias of pregnancy,

presented entirely different pictures. Case 26515 had electrolyte findings quite similar to those encountered after pyloric or upper intestinal obstruction, with an extreme reduction of chloride. Case 35556 had normal chlorides, but base and bicarbonate were both low. In this case the administration of 1000 cc. of normal saline had no appreciable immediate effect on either chloride or bicarbonate. The undetermined acid is quite low, so that organic acid cannot be held responsible for the reduction of bicarbonate. It is difficult to explain such diverse reactions on the basis of vomiting alone. A careful study of the inorganic metabolism during the development of the condition is essential. The majority of patients who enter the hospital for vomiting are so seriously ill as to require emergency treatment that interferes with adequate study.

#### *Esophageal Obstruction.*

Case 29239 had almost complete esophageal obstruction, which had prevented him from eating but had not caused him to vomit. Chloride in this case was low while bicarbonate was above the usual normal limits. The patient was seriously dehydrated when he entered the hospital but, by the time the blood was examined, had already had several saline hypodermoclyses. If these were in any sense effectual the chlorides of the plasma must have been even lower at the time of his admission to the hospital. When adequate feeding was effected through the gastrostomy the chlorides rose to an unusual level without any compensatory drop of bicarbonate. This could hardly have been possible unless base had also risen. It is apparently possible to develop an electrolyte picture similar to that of pyloric obstruction in esophageal obstruction. Vomiting of acid can have played no part in the production of the changes in this case because the patient did not vomit. Haden and Orr (2) have reported similar results after experimental obstruction of the cardiac end of the stomach. Although vomiting of hydrochloric acid is undoubtedly a factor in the depletion of the serum chloride it is not an essential factor. It may be that fasting is a determining cause.

The next three patients had intestinal obstruction due to different conditions. Case 29658 had an irreducible umbilical hernia; Case 34777 obstruction and infection of the bile ducts with para-

lytic ileus; and Case 34602 peritonitis with paralytic ileus due to rupture of an infected gall bladder. All had vomited profusely. Two out of the three had low chlorides and base was also reduced in the one instance in which it was determined.

### *Tetanus.*

Case 34053, a patient with tetanus studied shortly before death, had, as might have been expected, a rather high concentration of organic acid in his plasma. He had received sufficient glucose by hypodermoclysis to prevent ketosis. It is quite probable that the acid was lactic acid and was referable to abnormal muscular activity. The high chloride may have been due to the saline which he received with the glucose subcutaneously.

### *Lead Colic.*

The reason for the high organic acid of Case 32226 is not so clear. This patient had lead colic of such severity that it had produced complete obstipation for 3 days before the first examination. Although there was evident intestinal obstruction chloride was little depressed and what little alteration there was may have been due to the high organic acid. Base was somewhat increased. At the time of the second examination, when symptoms had disappeared, the organic acid, though still a little greater than normal, had receded considerably. Compensation had been effected by an increase of chloride and a diminution of base.

### *Infection.*

The next six cases had infections of various kinds. Bicarbonate is somewhat below normal in the group as a whole. This may well represent the effect of fever. Elevation of temperature reduces the bicarbonate capacity of the blood. The temperature coefficient of the carbon dioxide absorption curve has not as yet received sufficient attention to permit an accurate estimation of the effect of changes in temperature on the  $\text{CO}_2$ -combining capacity; but some studies made in this laboratory lead us to believe that it is considerable. The conservative reaction to fever should, therefore, be to reduce the height of the absorption curve. The absorption curve experiments in this series indicate that such a

change has taken place. In two instances only were chlorides also reduced. Both Patients 18725 and 33564 were unconscious and had received little or no food or fluids. Both showed abnormal muscular activity. Case 18725 was quite restless and had convulsive seizures at intervals. Case 33564 had severe choreiform movements. These movements together with a starvation ketosis may be responsible for the high organic acid presented by the latter patient.

### *Lobar Pneumonia.*

The six patients with pneumonia in the next group offer a striking contrast to those of the preceding series with other types of infections. In pneumonia the general level of bicarbonate is normal while chloride is low. The "total" acid is therefore somewhat reduced. Hypochloremia has long been recognized as a characteristic of pneumonia, but no adequate explanation of the chloride deficiency has been offered. It is known to be associated with a diminished urinary chloride excretion.

In only one case of this series, Case 35139, was total base low. In this instance the chloride reduction is only an indication of a total electrolyte deficiency. In the last three observations in the group (Cases 35762 and 35686) the chlorides have apparently been displaced by organic acid which appears in excessive amounts. Palmer (6) noted an excessive amount of organic acid in the urine of a certain proportion of patients with lobar pneumonia. Neither excessive organic acid nor deficiency of base seems to be a constant characteristic of the disease and therefore neither can be considered an essential factor in the reduction of the chlorides although either or both may be effective in individual cases.

The state of the acid-base equilibrium in pneumonia has been a source of considerable controversy. Means and his associates (1), from a comparison of the carbon dioxide content and absorption curves of arterial blood, came to the conclusion that uncompensated acidosis was frequently encountered in pneumonia. The temperature corrections which they employed were neither of the proper magnitude nor the proper sign. Hastings, Neill, Morgan, and Binger (3), employing more accurate methods and correction factors, found no evidence of acidosis of any kind in a study of a considerable number of pneumonia patients. The pH values

which we have obtained, although uncorrected for temperature, are so far above the lower limit of the normal range that temperature correction, which amounts to about 0.01 of pH for each degree of temperature, could not reduce them to a sufficient degree to produce an acidosis. On the basis of this work and theoretical considerations Hastings, Neill, Morgan, and Binger (3) and Van Slyke (5) have concluded that "in any individual breathing the ordinary atmosphere, respiratory hindrance must result in serious anoxemia long before  $\text{CO}_2$  acidosis has become at all significant." Earlier in the study of pneumonia by Hastings *et al.* this statement appears in italics: "the respiratory mechanism is unimpaired in its ability to maintain a normal  $\text{CO}_2$ -tension and blood reaction."

If any deductions can be drawn from a comparison of pneumonia with other febrile infections this statement may require modification. Attention has been called to the fact that a normal bicarbonate at  $38^\circ\text{C}$ . is probably equivalent to a higher bicarbonate at a greater temperature. Although the data in this paper are too scanty to permit more than tentative deductions, it is worthy of note that the  $\text{CO}_2$  of the pneumonia cases, although within the normal limits for afebrile subjects, is significantly higher than that of patients with fever due to other infections. This may be an indication that bicarbonate is relatively increased, but that the increase is masked by the opposing temperature effect. This would be in effect a compensated carbon dioxide acidosis.

The mechanism for the elimination of  $\text{CO}_2$  is overloaded even if it can and does maintain a normal  $\text{CO}_2$  tension and blood reaction. There can be no other explanation of the fact that pneumonia patients overventilate constantly without reducing the carbon dioxide tension or the carbon dioxide content of the blood below normal. Under these circumstances the best protective reaction against acidosis would be to increase the base bound by bicarbonate. Just such a reaction has been reported by Scott (7) in emphysema and has been corroborated by Essen, Kauders, and Porges (8). The latter have shown that the high blood bicarbonate concentration of emphysema is compensated by a chloride deficiency.<sup>1</sup>

<sup>1</sup> This observation has been confirmed by the present authors.



TABLE I  
Total Acid-Base Equilibrium of the Plasma in a Variety of Pathologic Conditions.

Case No.	Date.	Oxygen capacity:		Cell volume.		Ratio of Columns 1:2		Plasma.							Treatment of blood.	Remarks.
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)		
		vols. per cent	vols. per cent		Protein.	vols. per cent	mg. per 100 cc.	mg. per 100 cc.	mm	mm	mm	pH	mg. per 100 cc.			
34622	Sept. 19, 1924				6.41s	65.4	363	4.8	143.9	144.6	0.7		22	V. cont.		Phenol poisoning.
34909	Oct. 24, "	18.5	43.9	42.2	6.13s	58.0	382	4.2	145.1	156.3	11.2		44	"		Vomiting.
26515	Jan. 12, "	17.0	34.7	49.0	6.30	53.2	282		116.0			7.54	40	" cap.		Psychoneurosis. Pernicious vomiting.
35556	Dec. 31, "				8.61s	26.6	373	2.4	132.4	135.6	3.2		42	" cont.		Toxemia of pregnancy.
29239	Feb. 18, "	15.9	34.9	45.6	5.89	70.7	336	4.3	138.0			7.46	44	A. "		Vomiting.
	Mar. 1, "	17.9	37.5	47.7	5.98	73.5	391	2.9	155.1			7.60		"		Esophageal carcinoma.
29658	Mar. 19, 1924	20.6	46.4	44.4	7.97	61.4	316	4.9	138.2			7.50	41	V. cont.		"
																Irreducible umbilical hernia. Intestinal obstruction.

34777	Oct. 6, 1924	18.6			5.52s	64.9	312	2.6	126.4	139.2	12.8	80	V. cont.	Obstruction of common bile duct. Infectious cholangitis. Cholecystitis. General peritonitis.
34602	Nov. 17, "				6.40s	59.3	357	2.6	138.3			44	"	
34053	July 22, 1922	20.1	42.1	47.7	7.70	45.9	396	2.4	146.7	170.8	24.1	7.48*	V. cont.	Tetanus.
32226	" 12, 1924	12.8	28.2	45.4	7.33	60.3	348	2.2	138.0	171.7	33.7	7.41	"	Chronic plumbism.
	" 25, "	11.2	31.1	36.0	7.06	60.9	370	3.3	144.2	168.0	23.8	7.37	"	Lead colic.
33503	June 8, 1924	14.1	31.0	45.3	6.41	52.4	351	4.0	134.3	151.8	17.5		V. cont.	Abscess of breast.
26343	Nov. 13, 1923	15.9	38.2	41.5	7.01	59.2	353	138.9			7.40	29	" cap.	Lactosuria.
18725	May 26, "	20.1	43.3	46.4	8.27	49.9	355	131.6			7.32		"	Acute mediastinitis.
33564	June 15, 1924	15.3	36.0	42.4	7.03	46.4	338	3.8	129.4	157.7	23.3	7.41	" cont.	Hemorrhagic pachymeningitis. Coma.
15712	Feb. 17, 1923	21.2	43.6	48.6	7.66	50.3	374	141.5			7.32	33	" cap.	Pregnancy. Acute chorea. Coma.
10744	Nov. 11, 1922	16.2	40.9	39.6	6.77	43.1	392	139.6			7.25	51	"	Carbon monoxide poisoning. Influenza pneumonia.
15703	Feb. 17, "	20.1	42.1	47.7	6.62	58.5	346	135.8			7.39	59	"	Acute tracheitis. Bronchopneumonia. Fibrous pericarditis.
26550	Dec. 7, 1923	17.0	38.8	43.9	6.94	54.7	334	141.9			7.40*		" cont.	Lobar pneumonia.
35608	Jan. 5, 1925	9.9	31.9	31.1	5.82	59.2	344	3.5	133.9	149.4	15.5	27	"	"

TABLE I—Concluded.

Case No.	Date.	Oxygen capacity.		Cell volume.		Ratio of Column 1, 2.		Plasma.								Blood non-protein nitrogen.	Treatment of blood.	Remarks.
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)						
		vols. per cent.	vols. per cent.		per cent.	vols. per 100 cc.	mg. per 100 cc.	Cl	mg. per 100 cc.	Inorganic P.	Total acid.	Total base.	Organic acid.	pH				
35139	Nov. 15, 1924	20.5	50.3	40.9	5.90	66.2	316	4.6	131.8	137.8	6.0	7.63			V. cont.	Lobar pneumonia.		
35762	Jan. 27, "	15.8	30.3	52.2	5.93	50.7	318	2.9	122.8	155.1	32.3				"	"		
	Feb. 2, "	14.1	35.3	40.0	6.19	66.5	335	5.3	136.3	160.2	23.9				"	"		
35686	Jan. 19, "		44.7		5.87	55.4	349	3.2	132.8	157.9	25.1				"	"		
18859	June 9, 1923	3.6	11.0	32.4	6.97	53.3	373		141.6					7.37	V. cap.	Adenomyoma of uterus.		
22114	July 1, "	3.7	9.4	39.5	5.52	54.4	382		142.1					7.36	"	Secondary anemia.		
8169	June 2, "	2.9	6.1	47.8	6.57	47.9	366		136.3					7.30	"	Adenomyoma of uterus.		
33106	Oct. 14, 1924				5.42s			3.2		156.4					" cont.	Anemia.		
22780	Sept. 18, 1923	27.6	61.0	45.2	6.74	61.9	355		140.2					7.41*	"	General anasarca.		
	Oct. 1, "	27.8	65.5	42.5	7.23	48.8	383		142.7					7.31	" cap.	Polycythemia vera.		

<sup>s</sup> after the protein values in Column 4 indicates that serum instead of plasma was employed for analysis.

\* pH values followed by \* are 0.0 to 0.2 too high because the indicator solution used for these determinations was too acid.

Cf. discussion of Methods (4).

A and V in Column 13 indicate arterial and venous blood respectively; *cont.*, that the specimen was analyzed as drawn; *cap.*, that it was brought into equilibrium with 40 mm. of CO<sub>2</sub> in air at 38°C. before analysis.

If the hypothesis which has been suggested is correct, it offers at least a partial explanation of the low chloride of pneumonia which may be considered as a reaction to a compensated carbon dioxide acidosis. This would also explain the fact that reduction of base in one instance and an excess of organic acid in three others led to a further displacement of chloride instead of the reduction of bicarbonate which would ordinarily have been expected.

These remarks and explanations concerning the mechanism that determines the chloride reduction in pneumonia are built on insufficient data and are advanced tentatively. Further investigation of the subject is proposed as soon as suitable material again presents itself.

#### *Anemia and Polycythemia.*

The last five patients in Table I were suffering from diseases of the hematopoietic system. The first four had profound anemias, the last a polycythemia. There is nothing in the findings to indicate that anemias or polycythemias have any significant effect on the concentration of the electrolytes of the plasma.

#### SUMMARY AND CONCLUSIONS.

The effects of vomiting on the electrolytes of the serum are highly variable and probably depend on the nature of the vomitus, the severity and duration of emesis, and the degree of inanition produced. The most frequent result is a reduction of chloride, with or without a diminution of base. The level of bicarbonate is capricious.

Vomiting of hydrochloric acid is not essential for the production of this picture, as it has been encountered in a patient with esophageal obstruction who had not vomited.

In a series of infections bicarbonate was generally at or a little below the usual level. This may represent the reaction to temperature which reduces the carbon dioxide capacity of the blood.

In lobar pneumonia, on the other hand, bicarbonate is usually normal while chloride is almost invariably low. In some cases diminution of base and in others the presence of an unusual amount of organic acid are factors in determining the low serum Cl. These changes are, however, inconstant and therefore cannot be the essential causes of the characteristic hypochloremia. It is

tentatively suggested that the reduction of bicarbonate which usually occurs with elevation of temperature may be prevented in pneumonia by the impairment of the respiratory mechanism.  $\text{CO}_2$  is therefore maintained at a relatively high tension to facilitate escape of  $\text{CO}_2$  in the lungs and bicarbonate assumes a larger share of the blood base at the expense of chloride to minimize the reduction of pH that would otherwise ensue.

Anemia and polycythemia have no characteristic influence on the base or acids of serum.

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#### PROTOCOLS.

*Case 34622.*—French girl, age 18, waitress, single, admitted to the hospital the night of September 18, 1924. About half an hour earlier she had taken 2 tablespoonfuls of concentrated carbolic acid, but had vomited at once and had received gastric lavage with dilute alcohol followed by sodium bicarbonate. During the night she vomited several times food remains and bile-stained material, although she was given nothing but water by mouth. The blood was taken early the following morning. At this time the urine contained considerable phenol and a trace of albumin. The blood count

showed 4.1 million red blood cells and 10,900 leucocytes. She complained of thirst, but showed no signs of shock or dehydration. Recovery was rapid and complete. *Impression:* Phenol poisoning.

*Case 34909.*—American, female, age 27, single, factory forewoman, admitted to the hospital October 20, 1924. For 7 years she had been troubled with nausea and vomiting after almost every meal and for a year and a half had also had attacks of pain in the right lower quadrant of the abdomen. She appeared quite thin, but not wasted, highly nervous, and sensitive. Dietetic treatment and medication gave her no relief. After almost every meal she vomited. Evidently only part of each meal was returned because her urine showed no acetone or diacetic acid and she did not lose weight at an excessive rate. No free hydrochloric acid and reduced amounts of total acid were found repeatedly in vomitus, fasting gastric contents, and gastric contents after a test meal. General physical and special examinations were negative; but symptoms subsequently disappeared after exploratory laparotomy. The blood was examined before breakfast, October 24, when she had been in the hospital 4 days and was vomiting constantly. She did not appear dehydrated. *Impression:* Psychoneurosis; persistent vomiting; hypochlorhydria.

*Case 26515.*—American, female, age 29, in the early part of the 4th month of pregnancy, admitted to the hospital December 21, 1923. About a month before this she began to suffer from nausea and vomiting which soon became so serious that she was unable to retain any food at all. When she came to the hospital she appeared dehydrated and moderately toxic, and the urine contained a trace of albumin and considerable acetone and acetoacetic acid. The phenolsulfonephthalein excretion was 60 per cent. The blood was examined before breakfast January 12. Vomiting persisted and she had received saline and glucose by rectum and subcutaneously. The blood pressure was not elevated; the retinae showed no abnormalities; the liver was neither enlarged nor tender; there was no evidence of edema. Her urine contained a slight trace of albumin, no sugar, traces of acetone and acetoacetic acid, and a few red blood cells. *Impression:* Toxemia of pregnancy with pernicious vomiting.

*Case 35556.*—American, female, age 25, married, admitted to the hospital, December 30, 1924, in the 3rd month of pregnancy. For 7 weeks she had vomited several times every day, with the exception of one short period of 5 or 6 days. She appeared somewhat toxic, her skin and mucous membranes were dry, the tongue coated, the respirations rather deep and somewhat labored. There was no subcutaneous edema. Ophthalmoscopic examination was negative. The urine contained considerable acetone and some urobilin, but no albumin or sugar. Blood count, 5.2 million red blood cells, 20,000 leucocytes. Rosenthal's phenoltetrachlorophthalein test for liver function showed 10 per cent of the dye still in the serum at the end of an hour and a half, indicating considerable impairment of liver function. Blood was examined just before and just after a hypodermoclysis containing 500 cc. of 5 per cent glucose solution and 1000 cc. of normal saline. Sub-

sequent therapeutic abortion was followed by complete recovery. *Impression:* Toxemia of pregnancy with pernicious vomiting.

*Case 29239.*—English, male, age 57, admitted to the hospital, February 15, 1924. Since the latter part of December he had been unable to swallow solid food at all and for about 2 weeks before admission had had only milk. In spite of the severity of his dysphagia he had vomited only once in the course of his illness. He appeared, however, quite emaciated and dehydrated. Examination revealed almost complete obstruction of the esophagus. The first blood examination was made the morning of February 18, and gastrostomy was performed later in the same day. Between the time when he entered the hospital and the time of the venous puncture he received two hypodermoclyses of 1500 cc. of saline each, the last only an hour or two before the blood was withdrawn. The second examination was made March 1, when he was receiving a satisfactory amount of nourishment through the gastrostomy opening.

*Case 29658.*—An old Irish woman, admitted to the hospital March 19, 1924. 36 hours earlier an umbilical hernia, which had been gradually increasing in size for 11 or 12 years, suddenly became painful, hard, and irreducible. She became nauseated and vomited a little later. At the time of admission she appeared thin and dehydrated, mentally confused, quite toxic. Her temperature was 100°F. The pulse was totally irregular and there was evident auricular fibrillation. The systolic blood pressure was 160, diastolic 90. The blood was examined at once. Urine obtained at the same time showed a specific gravity of 1.024, a faint trace of albumin, no sugar, many hyaline casts, many leucocytes, and an occasional red blood cell. Non-operative reduction of the hernia was successful. *Impression:* Incarcerated umbilical hernia; intestinal obstruction.

*Case 34777.*—German, female, age 53, admitted to the hospital October 5, 1924. Cholecystectomy had been performed in 1920. On September 27 she was seized with nausea and vomiting which persisted till she entered the hospital. She was intensely jaundiced, somewhat stuporous; temperature 101.5°F., pulse 110, respirations 44 to the minute. The urine contained considerable bile, the stools none. The blood was examined a few hours later, when her condition was little altered. Meanwhile she had received only 400 cc. of water by mouth and 1600 cc. of normal saline solution by hypodermoclysis and had vomited several times. Subsequent unsuccessful operation revealed obstruction of the common bile duct and infectious cholangitis.

*Case 34602.*—Hebrew, female, age 60, married, admitted to the hospital November 16, 1924. 3 days earlier she was suddenly seized with general abdominal pain, vomiting, and obstipation which persisted until she entered the hospital when she appeared quite jaundiced and dehydrated; temperature 98.6°F., pulse 105 and somewhat thready, respirations 28 to the minute. The whole upper half of the abdomen was rigid and tender. Her urine contained considerable albumin, bile, and urobilin, but no sugar; specific gravity 1.025. The blood was examined early on the morning of November 17, just before operation. She had received nothing by mouth

and only 800 cc. of normal saline solution by hypodermoclysis since admission and her condition was unchanged. Operation, which was successful, revealed gall stones, obstruction of the common and cystic ducts, and a ruptured gall bladder. *Impression:* Cholelithiasis; obstructive jaundice; peritonitis.

*Cases 34053.*—American, male, age 48, admitted to the hospital, July 19, 1924, with tetanus. The blood examination was made shortly before his death on July 23, when his temperature was 105.4°F., pulse 160, respirations 52, and he was having almost continuous clonic convulsions. In the preceding 24 hours he had received about 3000 cc. of glucose solution and normal saline intravenously and subcutaneously. *Diagnosis:* Tetanus.

*Case 32226.*—Russian, male, age 35, painter, admitted to the hospital July 12, 1924. For 3 days, in spite of catharsis, his bowels had failed to move and he had suffered from abdominal cramps. The first blood examination was made when he was admitted to the hospital. At this time there was general abdominal tenderness with palpable fecal masses in the intestine, a slight lead line on the gums. The systolic blood pressure was 120, diastolic 80. The urine was negative. The blood count showed 4.8 million red blood cells and 5,500 leucocytes. The erythrocytes showed definite stippling. At the time of the second examination, July 25, symptoms had been relieved and the bowels were moving regularly. *Impression:* Chronic lead poisoning; intestinal colic.

*Case 26503.*—American, female, age 33, admitted to the hospital June 8, 1924, 1 month post partum with an abscess of the right breast, a temperature of 103°F., pulse 130, respirations 30. The blood examination was made immediately. The urine at this time contained a trace of sugar unfermentable by yeast and small amounts of acetone and acetoacetic acid. The blood sugar was only 135 mg. per cent. *Impression:* Abscess of breast; lactosuria.

*Case 26343.*—Italian, female, age 48, admitted to the hospital November 12, 1923. She appeared seriously distressed, slightly cyanotic, sitting up in bed because of the pain and dyspnea; temperature 102°F., pulse 120, respirations 36. The superficial veins over the upper part of her chest appeared distended. The pharynx and tonsils were somewhat inflamed. At the base of the right chest resonance, voice, and breath sounds were somewhat diminished. Pressure over the sternum was quite painful. X-ray of the chest revealed increased density in the superior anterior mediastinum. The blood was examined before breakfast November 13, when her condition was entirely unchanged. Her temperature was still 102°F. *Impression:* Acute mediastinitis.

*Case 18725.*—Irish saloon-keeper, age 38, admitted to the hospital May 5, 1923. That morning he was found unconscious and later had several generalized convulsions. 2 years earlier he had had a series of convulsive seizures. Ophthalmoscopic examination revealed slight choking of both optic discs. His temperature was 102°F., pulse 98; systolic blood pressure 160. The liver was distinctly enlarged, with a smooth, firm edge. There were no paralyses and all tendon reflexes were active. Shortly after



admission he had a short convulsive seizure, beginning in the right leg. The blood Wassermann was negative. The spinal fluid was bloody. The urine showed only a few hyaline casts. The blood was examined May 26, before breakfast. The patient appeared somewhat more rational and improved, but had another convulsion in the course of the morning. His temperature was 101°F. Autopsy, 5 days later, revealed a hemorrhagic pachymeningitis and gangrene of the lung.

*Case 33564.*—Irish girl, age 19, married, admitted to the hospital June 14, 1924, 5 months pregnant. 2 months earlier she had become nervous and irritable, awkward in the use of her hands, and had developed peculiar muscular twitchings which increased steadily. On the day of admission she gradually sank into deep coma, and for the first time was found to have a high temperature. When first seen in the hospital her temperature was 103.4°F., pulse 160 to the minute, respirations 45, systolic blood pressure 129. She was in deep coma, moaning, restless, and twitching constantly. The skin and mucous membranes were extremely dry and slightly cyanotic. There was slight rigidity of the neck and a questionable Kernig's sign and loss of deep tendon reflexes. A systolic murmur and thrill were preceptible at the cardiac apex. Blood culture, Wasserman test, and spinal fluid were normal. Blood count showed 5.2 million red blood cells and 12,400 leucocytes. Blood was examined the morning of June 15, when her temperature was 104°F. She had taken only about 500 cc. of water and no food meanwhile. While she was in the hospital only 450 cc. of urine could be obtained by catheter. This contained a large amount of albumin, traces of sugar and acetone, and many granular casts. *Diagnosis, confirmed by autopsy:* Chorea, acute endocarditis.

*Case 15712.*—American, male, age 26, admitted to the hospital February 17, 1923. The previous day he suddenly developed headache, sore throat, and fever. At 2.30 the next morning he was found unconscious on the bathroom floor, a gas heater beside him disconnected, with the gas turned on. On admission he was unconscious, breathing rapidly, but cyanotic; temperature 106°F., pulse 118, respirations 30. Breath sounds were everywhere harsh and râles were heard all over the chest. Wassermann, blood culture, and lumbar puncture were negative. Blood was taken for examination immediately after he entered the hospital. It contained 1.74 volumes per cent of carbon monoxide. The patient ran a typical course of severe influenzal bronchopneumonia with complete recovery. *Impression:* Carbon monoxide poisoning; influenzal bronchopneumonia.

*Case 10744.*—American, female, age 42, admitted to the hospital November 10, 1922. 2 weeks earlier she developed intense occipital headache, general myalgia, and high fever. 3 days before admission the headache became more severe and the next day she had a generalized convulsion that lasted about half an hour. Shortly before she came to the hospital she had a second convulsion. On admission she was in a semicomatose condition, breathing rapidly and stertorously, with definite orthopnea and moderate cyanosis. Coarse, moist râles and harsh breath sounds were audible over the whole chest. Systolic blood pressure was 165; temperature was ele-

vated. Blood culture, ophthalmoscopic and otoscopic examinations and spinal fluid were negative. Blood non-protein nitrogen was 51 mg. per cent. Urine, obtained by catheter, showed considerable albumin, no sugar, and only a few casts. The blood count was: red blood cells 4.8 million, leucocytes, 28,900. It was impossible to make the patient take fluids by mouth and the total urine output while she was in the hospital amounted to only 800 cc. The blood examination was made before breakfast November 11. Her temperature at this time was 103°F. Autopsy next day revealed acute tracheitis, bronchopneumonia, and fibrous pericarditis.

*Case 15703.*—English laborer, age 54, admitted to the hospital on February 15, 1923. The preceding day he had a sharp chill, followed by severe cough and pain in the right side of the chest and the expectoration of bloody sputum. On admission he was cyanotic, toxic, and breathing rapidly and with a distinct effort. His temperature was 104°F., pulse 120, respirations 30 to the minute; systolic blood pressure 120, diastolic 45. The upper lobe of the right lung was consolidated. Blood count: leucocytes 24,300 with 88 per cent of polymorphonuclear neutrophils. The blood was examined February 17 when his temperature was 103.5°F. The next day his temperature fell by crisis. *Impression:* Lobar pneumonia.

*Case 26550.*—American storekeeper, age 46, admitted to the hospital December 6, 1923. 5 days before admission he noticed a dull pain in the right chest, a productive cough, and fever. At the time of admission his respirations were rapid (35 to the minute) and shallow and he was slightly cyanotic; temperature 103°F. There was frank consolidation of the right lower lobe. Blood culture was sterile; the sputum yielded pneumococcus, Type IV. The urine contained a moderate amount of albumin and a few granular casts. The blood was examined the next morning when the temperature was 104°F. *Impression:* Lobar pneumonia.

*Case 35608.*—American, female, age 26, admitted to the hospital January 5, 1925. For 2 months she had been losing weight and strength. January 1 she had a chill and the next day developed pain in her right chest and a high fever. The symptoms increased steadily until she entered the hospital. On admission she appeared extremely toxic, quite pale, but cyanotic, with considerable dyspnea. There was dried blood in the anterior nares, lips, and pharyngeal wall. The lower lobes of both lungs were consolidated. Blood culture yielded Type I pneumococci; Wassermann test negative. Blood count showed 2.7 million red blood cells and 23,500 leucocytes with 89 per cent of polymorphonuclear neutrophils. The temperature was 105°F., pulse 120, respirations 54 to the minute. The blood examination was made almost as soon as the patient entered the hospital. She died the next day and autopsy was refused so the cause of her profound anemia was not discovered.

*Case 35139.*—Colored, male, age 21, admitted to the hospital November 13, 1924. 1 week earlier (November 6) he developed a sharp pain in the left side of his chest, with fever and a productive cough. Symptoms continued to increase in severity until he entered the hospital. On admission he appeared prostrated, quite breathless, suffering from considerable pain

and cough with purulent expectoration. There was a definite friction rub over the left chest and the lower lobe of the left lung and the upper lobe of the right lung were consolidated. The abdomen was tense and there was tenderness in both upper quadrants. Blood culture remained sterile. The sputum yielded a Type II pneumococcus. The blood count showed 5.6 million red blood cells and 41,300 leucocytes with 96 per cent of polymorphonuclear neutrophils; the temperature was 102°F., pulse 140, respirations extremely rapid. The blood was examined on the morning of November 15, 2 days later, when the patient's condition was little changed; temperature 101.6°F., pulse 120, respirations still extremely accelerated. He recovered completely.

*Case 35762.*—American, male, age 35, admitted to the hospital January 26, 1925, on the 2nd day of a typical lobar pneumonia involving the right upper lobe, moderately toxic, dyspneic, and somewhat cyanotic. Blood culture was sterile; sputum yielded a Type II pneumococcus. Blood count showed 4.7 million red blood cells and 22,000 leucocytes with 96 per cent of polymorphonuclear neutrophils. Temperature 105°F., pulse 134, respirations 30. The blood was first examined the next morning. Meanwhile the patient had developed auricular fibrillation. His temperature was still 104.5°F., respirations 40, and cyanosis was more marked. The temperature fell by lysis from January 29 to February 3. At the time of the second examination, on February 2, the temperature was only 100°F.

*Case 35686.*—Male, age 30, admitted to the hospital January 17, 1925. 4 weeks earlier he had developed a mild, productive cough. The night before admission he had a slight chill, followed by breathlessness and pain in the right side of the chest. 4 years before this he had pleurisy and an anal fistula, and in July, 1923, a small hemoptysis. At the time of admission his temperature was 103.6°F., pulse 120, respirations 30 to the minute, labored and painful. There was moderate cyanosis. The lower lobe of the right lung was frankly consolidated. Blood count: 5 million red blood cells, 37,000 leucocytes, with 95 per cent polymorphonuclear neutrophils. Blood culture was sterile; hemolytic streptococci predominated in the sputum. Subsequently tubercle bacilli were found. Blood was examined on January 19. At this time the general condition seemed improved, but the signs in the right chest had extended and cyanosis was more marked. The temperature was 101°F., pulse 100. The urine contained a trace of albumin and a few hyaline and granular casts. Subsequently the patient developed a transient pericarditis and then empyema which required thoracotomy. *Impression:* Pulmonary tuberculosis; streptococcus pneumonia.

*Case 18869.*—American, female, age 48, admitted to the hospital June 8, 1923. For 4 years her menstrual periods had occurred every 2 weeks with an excessive loss of blood at each period. On admission she appeared pale. Her pulse was rapid, respirations and temperature normal. The uterus was retroverted, enlarged, and nodular. The urine was negative. There was no subcutaneous edema. Blood count: red blood cells, 3.2 million, leucocytes, 7,300 with 69 per cent of polymorphonuclear neutrophils. The blood examination was made before breakfast the next morning when her

condition was essentially unchanged. Hysterectomy preceded by transfusion resulted in complete recovery. *Impression:* Myomata of uterus; secondary anemia.

*Case 8169.*—American, female, age 47, with pernicious anemia, admitted to the hospital June 1, 1923, for transfusion. She had slight subcutaneous edema of the trunk and extremities. Blood count: red blood cells 0.66 million, leucocytes 1000, with only 28 per cent of polymorphonuclear leucocytes. The blood was examined the next morning just before she received a blood transfusion. *Impression:* Pernicious anemia.

*Case 22114.*—American, female, age 48, admitted to the hospital June 30, 1923. 3 years earlier she developed a severe anemia that cleared up under treatment in the course of a month. 6 weeks before admission she noticed that she was becoming steadily weaker and paler. For 7 years she had been troubled with bleeding from the vagina at irregular intervals, often passing large clots.

On admission she was extremely pale and sallow and weak, breathing rather rapidly and superficially and complaining of throbbing headache. There was no atrophy of the mucous membrane of the tongue, but the gastric contents contained no free hydrochloric acid. The liver edge could be felt at the level of the umbilicus, but the spleen did not appear to be enlarged. A large bleeding polyp protruded from the uterine cervix. The blood examination was made the next morning just before she received a blood transfusion. The blood count at this time showed 1.1 million red blood cells and 8,200 leucocytes, with 67 per cent of polymorphonuclear neutrophils and a few nucleated red cells. The patient had slight edema of the lower extremities, but the urine showed no significant changes. In spite of transfusion and hysterectomy she died 4 months later. *Impression:* Profound anemia, possibly primary.

*Case 33106.*—German, female, age 52, with pernicious anemia, admitted to the hospital for transfusion. When the blood was examined it contained only 0.6 million red blood cells, 19.5 per cent of hemoglobin, 4,700 leucocytes, with 40 per cent of mononuclear cells and no nucleated red blood cells. The patient presented extreme dyspnea and orthopnea, generalized subcutaneous edema, double hydrothorax, and ascites. *Impression:* Primary anemia; general anasarca.

*Case 22780.*—Russian Hebrew, female, age 29, admitted to the hospital September 12, 1923. For 18 months she had suffered increasingly from headaches, attacks of vomiting, and frequency of urination. She appeared well developed and nourished, somewhat florid. Her systolic blood pressure was 200, her urine contained a trace of albumin and occasional casts and the phenolsulfonephthalein excretion was 70 per cent in 2 hours and 10 minutes. The first blood examination was made September 18, before breakfast. At this time her blood count was: red blood cells 8.4 million, leucocytes 16,500 with 68 per cent of polymorphonuclear neutrophils. The second examination was made October 1, when the blood count was practically unaltered: red blood cells 8.4 million, leucocytes 13,000 with 79 per cent of polymorphonuclear neutrophils. *Impression:* Polycythemia rubra.



## THE APPARENT DISSOCIATION CONSTANTS OF CREATINE AND CREATININE.

By GEORGE S. EADIE AND ANDREW HUNTER.

(From the Department of Biochemistry, University of Toronto, Toronto,  
Canada.)

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The extent to which the hydrochlorides of creatine and creatinine undergo hydrolytic dissociation has been estimated by Wood (1) from their catalytic effect upon the saponification of methyl acetate, by Hahn and Barkan (2) from the hydrogen ion concentration of their aqueous solutions as determined by the gas chain. Wood's results led to basic dissociation constants of  $1.81 \times 10^{-11}$  for creatine and  $3.57 \times 10^{-11}$  for creatinine, Hahn and Barkan's to  $4.8 \times 10^{-12}$  for the first and  $1.85 \times 10^{-10}$  for the second. The constants of Wood, it should be said, are given for a temperature of  $40.2^\circ\text{C}$ ., the others for  $16-17^\circ$ . Since the two sets of results are far from consistent, and since the methods upon which they depend are subject to considerable experimental error, it seemed worth while to attempt a determination of the apparent dissociation constants of both bases by the method of electrometric titration.

The creatine employed was prepared from commercial creatine. A solution of this material in fifteen parts of hot water was boiled for a short time with a little animal charcoal, filtered rapidly under suction, mixed with twice its volume of alcohol, and allowed to crystallize in the ice box. The crystals were collected on a Buchner funnel, washed with a little 65 per cent alcohol, and drained as completely as possible. They were then, while still moist, redissolved by heating in two-fifths of the quantity of water originally used. The solution was cooled and the second crop of crystals was collected as before, washed with small quantities of ice-cold water, and allowed to dry in the air. The yield was 85 per cent of the quantity originally taken. The purity of the product was

indicated by the fact that 2.4456 gm., when dried to constant weight at 110°, lost 0.2956 gm. This implies a water content of 12.09 per cent, which is the theoretical amount for  $C_4H_5O_2N_3$ ,  $H_2O$ .

Our creatinine also was obtained from commercial creatine.

TABLE I.  
*Titration of 50 Cc. of 0.0891 M Creatinine.*

0.2 N HCl added.	0.2 M creatinine left uncombined = 22.3 - (a).	pH	pOH = 14.07 - (c).	$\log \frac{A}{C-A}$ = $\log \frac{(a)}{(b)}$ .	pK' <sub>b</sub> = (d) - (e).
(a)	(b)	(c)	(d)	(e)	(f)
cc.	cc.				
0.0	22.3				
0.5	21.8	6.51	7.56	-1.64	9.20
1.0	21.3	6.17	7.90	-1.33	9.23
1.5	20.8	6.00	8.07	-1.14	9.21
2.0	20.3	5.85	8.22	-1.01	9.23
2.5	19.8	5.76	8.31	-0.90	9.21
3.0	19.3	5.68	8.39	-0.81	9.20
4.0	18.3	5.54	8.53	-0.66	9.19
5.0	17.3	5.38	8.69	-0.54	9.23
6.0	16.3	5.31	8.76	-0.43	9.19
7.0	15.3	5.21	8.86	-0.34	9.20
8.0	14.3	5.04	9.03	-0.25	9.28
9.1	13.2	5.00	9.07	-0.16	9.23
10.0	12.3	4.97	9.10	-0.09	9.19
11.0	11.3	4.90	9.17	-0.01	9.18
12.0	10.3	4.80	9.27	+0.07	9.20
13.0	9.3	4.72	9.35	+0.15	9.20
14.0	8.3	4.63	9.44	+0.23	9.21
15.0	7.3	4.60	9.47	+0.31	9.16
16.0	6.3	4.48	9.59	+0.41	9.18
17.0	5.3	4.41	9.66	+0.51	9.15
18.0	4.3	4.29	9.78	+0.62	9.16
20.0	2.3	3.99	10.08	+0.94	9.14

The method of preparation adopted was that one among those proposed by Edgar and Hinegardner (3) which involves the conversion of the dried creatine to creatinine hydrochloride by the action of gaseous HCl and the subsequent liberation of the base by treatment with aqueous ammonia. The product was purified

TABLE II.  
*Titration of 50 Cc. of 0.0544 M Creatine.*

V = total volume of titration mixture.  $A = \frac{0.2}{V} \times \text{cc. of } 0.2 \text{ N HCl added.}$

$$C = \frac{0.0544 \times 50}{V}$$

$$A' = A - [H']$$

$$\text{pOH} = 14.07 - \text{pH}$$

V		C		A	pH	$\frac{a_{H^+}}{\gamma[H^+]}$	$\gamma$	[H']	A	C - A'	$\log \frac{A'}{C - A'}$	pOH	pK'b
cc.	cc.	M	N										
0.0	50.0	0.0544											
0.5	50.5	0.0539	0.00198		4.50					0.0519*	-1.42*	9.57	10.99
1.0	51.0	0.0533	0.00392		4.14					0.0494*	-1.10*	9.93	11.03
1.5	51.5	0.0528	0.00582		3.99					0.0470*	-0.91*	10.08	10.99
2.0	52.0	0.0523	0.00769		3.84					0.0446*	-0.76*	10.23	10.99
2.5	52.5	0.0518	0.00952		3.67					0.0423*	-0.65*	10.40	11.05
3.0	53.0	0.0513	0.0113		3.57					0.0400*	-0.55*	10.50	11.05
3.5	53.5	0.0508	0.0130		3.52	0.00030	0.91	0.00033	0.0127	0.0381	-0.48	10.55	11.03
4.0	54.0	0.0504	0.0148		3.48	0.00033	0.91	0.00036	0.0144	0.0360	-0.40	10.59	10.99
5.0	55.0	0.0494	0.0182		3.30	0.00050	0.90	0.00056	0.0176	0.0318	-0.26	10.77	11.03
6.0	56.0	0.0486	0.0214		3.14	0.00072	0.89	0.00081	0.0206	0.0282	-0.13	10.93	11.06
7.0	57.0	0.0477	0.0246		3.04	0.00091	0.89	0.0010	0.0236	0.0241	-0.01	11.03	11.04
8.0	58.0	0.0469	0.0276		2.94	0.00115	0.89	0.0013	0.0263	0.0206	+0.11	11.13	11.02
9.0	59.0	0.0461	0.0305		2.86	0.00138	0.88	0.0016	0.0289	0.0172	+0.23	11.21	10.99
10.0	60.0	0.0453	0.0333		2.75	0.00178	0.88	0.0020	0.0313	0.0140	+0.35	11.32	10.97
11.0	61.0	0.0446	0.0361		2.67	0.00214	0.88	0.0024	0.0317	0.0109	+0.49	11.40	(10.91)
12.0	62.0	0.0439	0.0387		2.62	0.00240	0.87	0.0028	0.0359	0.0080	+0.65	11.45	(10.80)
13.0	63.0	0.0432	0.0413		2.55	0.00282	0.87	0.0032	0.0381	0.0051	+0.87	11.52	(10.65)
14.0	64.0	0.0425	0.0437		2.48	0.00331	0.87	0.0038	0.0399	0.0026	+1.19	11.59	(10.40)

\* In calculating these figures the A' of the column headings was replaced by A.



by recrystallization from aqueous acetone as Edgar and Hinegardner suggest.

For the titration 0.8110 gm. of creatine hydrate (equivalent to 0.713 gm. of the anhydrous base) and 1.0078 gm. of creatinine were each dissolved in water sufficient to make a total volume of 100 cc. The molar concentrations of these solutions were 0.0544 and 0.0891 respectively. Of each a 50 cc. portion was submitted in the usual way to electrometric titration with 0.2 N HCl, the titration vessel and its contents being maintained by a water thermostat at a temperature of  $20 \pm 0.1^\circ\text{C}$ . For the calculation of pH from potentiometer readings we made use of the tables of Schmidt and Hoagland (4), which are based upon the data of Lewis, Brighton, and Sebastian (5).

The results of the two titrations, together with the calculations based upon them, are shown in Tables I and II.

The calculation of the dissociation constants of weak acids or bases from their titration curves rests upon the well known Henderson-Hasselbalch approximation, which in the case of a base takes the form

$$\text{pK}'_b = \text{pOH} - \log \frac{[\text{salt}]}{[\text{free base}]} \quad (1)$$

$K'_b$  is the "apparent" dissociation constant, which is related to the true dissociation constant,  $K_b$ , by the equation  $K_b = \gamma K'_b$ , in which  $\gamma$  is the "degree of ionization" of the salt or, if this is regarded as completely dissociated, the "activity coefficient" of its cation (6). Equation (1) involves as it stands the assumption that the free base is not appreciably ionized. If we were to add to this the assumption that  $\gamma$  is constant throughout the titration and equal to unity,  $K'$  would become identical with  $K$ . The approximation thus obtained is in many cases and for many purposes sufficiently close to the truth.

Provided (1) that the titrating acid is a strong one and (2) that the mixture undergoing titration possesses a reaction not too distant from neutrality (not more acid, say, than pH 3), the salt formed may be taken as the equivalent of the total acid added. If then the latter, expressed as gram equivalents per liter of the mixture, be denoted by A, and if the molecular concentration of the total base (combined or free) be represented by C, the con-

centration of free base will be  $C - A$  and the basic equation (1) may be written

$$pK'_b = pOH - \log \frac{A}{C - A} \quad (2)$$

To obtain the ratio which appears in the last term of this formula it is of course not necessary to calculate the actual concentrations within the mixture either of acid or of base; it is sufficient to express each in equivalent terms, say as volumes of  $N/5$  solution. It is in this manner that equation (2) has been used in Table I to calculate  $pK'_b$  for creatinine. The results, it will be seen, show a reasonable approach to constancy. Their average is 9.20, which corresponds to an apparent dissociation constant,  $K'_b$ , of  $6.3 \times 10^{-10}$ .

In the titration of the weaker base creatine the reaction becomes ultimately much more acid than pH 3. When this occurs it is no longer permissible to regard "salt" and "added acid" as equivalent. The phenomenon of "salt hydrolysis" now assumes importance and the mixture comes to contain not only salt and uncombined base, but also appreciable quantities of free acid. To learn in such a case the quantity of salt one must deduct from the acid added the quantity of acid remaining free. Upon the view now current that strong acids are completely ionized the concentration of the free acid will be given by the concentration of hydrogen ions. Accordingly, the symbols  $A$  and  $C$  being used in the same sense as before, the concentration of salt will now be  $A - [H^+]$ , that of uncombined base will be  $C - (A - [H^+])$ , and the basic equation will become

$$pK'_b = pOH - \log \frac{A - [H^+]}{C - (A - [H^+])} \quad (3)$$

which is the logarithmic form of an equation developed by Van Slyke (7).

In the application of a formula like the last it has been usual to assume that  $[H^+]$  can be determined directly by the hydrogen electrode, and to calculate it from the relation

$$\frac{E.M.F. \text{ (observed)} - E \text{ (calomel)}}{0.0001984 \text{ } T} = pH = -\log [H^+]$$

Now the potential difference measured by the gas chain depends in reality not upon the stoichiometric concentration of the hydrogen ion but upon its "activity." To obtain from electrometric data the true value of  $[H^+]$  we should therefore employ the more exact relation

$$pH = -\log a_{H^+} = -\log \gamma[H^+]$$

in which  $pH$  has the same *numerical* significance as before,  $a_{H^+}$  is the activity of the hydrogen ion, and  $\gamma$  is its activity coefficient. This relation we have made use of in Table II to calculate  $[H^+]$  in all but the earlier stages of the titration; and by inserting the values so found in equation (3) have obtained corrected estimates of  $pK'_b$ . As the appropriate value of  $\gamma$  at any given point of the titration we have taken the activity coefficient, as computed by graphical interpolation from the recent data of Scatchard (8),<sup>1</sup> of the hydrogen ion in a hydrochloric acid solution of concentration  $A$ . In doing so we assume the validity here of the principle of Lewis and Randall (9), that "in dilute solutions the activity coefficient of any ion depends solely upon the total ionic strength of the solution."

The procedure by which we have estimated the concentration of  $H^+$ , and therefore of free acid, in our titration mixtures is essentially the same as that followed by Hirsch (10); but Hirsch uses a uniform activity coefficient (or, as he calls it, "degree of dissociation") of 0.74, obtained, he states, from experimental measurements of the  $pH$  of hydrochloric acid solutions having a total  $Cl'$  concentration less than 0.1  $N$ . Neither Hirsch's method of calculation nor our own differs in form from one proposed still earlier by Harris (11); but the  $\alpha$ , which in Harris' formula takes the place of our  $\gamma$ , is the "degree of dissociation" of  $HCl$  as determined by conductivity measurements, and, as Cohn and Berggren (12) point out, it is erroneously assumed to be unaffected by the presence of another electrolyte. The inconsistency of applying conductivity data to the interpretation of potentiometric measurements and the importance of recognizing the effect of the total ionic concentration upon activity have recently been admitted by Harris himself (13).

<sup>1</sup> Scatchard's table contains one obvious error of computation; the coefficient for a molality of 0.02 should be 0.896, not 0.887.

In the earlier stages of the creatine titration  $[H^+]$  is so small that it may be neglected. For the first six steps therefore the constant has been calculated by equation (2). It may be added that it is only toward the end of the titration that the factor  $\gamma$  begins to have a really appreciable influence on the result. Until pH has fallen below 2.7 the simpler method of calculation followed by Van Slyke, in which  $[H^+]$  and  $a_{H^+}$  are assumed to be identical, yields values of  $pK'$  which hardly differ from those recorded in the table. As the acidity rises the correction introduced by  $\gamma$  becomes increasingly important; but even in the last term of the table it makes a difference in the apparent constant of only +0.10.

The correction is not large enough to prevent an obvious falling off in the last three or four values of  $pK'$ . We think it likely that this is due less to the failure of any of the assumptions underlying the calculation, than to an incipient transformation of the creatine into creatinine. Without attempting to elucidate the point we have decided arbitrarily to reject the aberrant values bracketed in the table, and to accept as the most probable result the average of the remainder. This would give to  $pK'_b$  for creatine the magnitude 11.02. The apparent dissociation constant is then  $9.6 \times 10^{-12}$ .

Our results differ very considerably from Wood's; but if they be compared with those of Hahn and Barkan it will be seen that, while they are by no means identical, they are in each case of the same order of magnitude. The difference may be partly accounted for by the higher temperature at which our determinations were made. If our estimates be accepted creatinine would appear to be 66 times stronger as a base than creatine. The ratio of the constants according to Hahn and Barkan is 38; according to Wood only 2. It would appear to be very doubtful whether Wood was dealing with pure substances.

#### SUMMARY.

Creatine and creatinine were titrated electrometrically with hydrochloric acid at 20°C. From the results the apparent basic dissociation constant ( $K'_b$ ) of creatine is calculated to be  $9.6 \times 10^{-12}$ , that of creatinine  $6.3 \times 10^{-10}$ ; or  $pK'_b$  for creatine is 11.02, for creatinine 9.20.

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# STUDIES ON THE CHEMICAL COMPOSITION OF BEEF BLOOD.

## I. THE CONCENTRATIONS OF CERTAIN CONSTITUENTS IN NORMAL BEEF PLASMA.\*

By C. S. ROBINSON AND C. F. HUFFMAN.†

*(From the Chemical Laboratory and Department of Dairy Husbandry of the Michigan Agricultural Experiment Station, East Lansing.)*

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For many years beef blood has served as the medium for abstract experimentation on blood. The methods of analysis which have been so highly developed during the past 10 years have been largely worked out on the blood from slaughtered cattle. Much of our fundamental knowledge of blood chemistry has been acquired from the same source. But recently in undertaking the study of the effect of certain restricted rations on such animals it was discovered that the data on the chemical composition of normal beef blood available for application to a study of the physiological processes of dairy cows were extremely meager. The studies hitherto pursued had dealt with beef blood merely as blood and had served only as a means to an end, the end being considerations other than the animals furnishing the blood.

Among the few who have secured information on the subject, Abderhalden (1) was among the first. He analyzed the bloods from several domestic animals, among them two beeves. Hart (2, 3, 4) and his collaborators have recorded a few determinations on calcium and phosphorus in blood and the changes in the concentrations of these two constituents under the influence of various factors affecting calcium assimilation. Meigs, Blatherwick, and Cary (5) have studied the calcium and phosphorus

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changes in the blood of cows, while Blatherwick (6) has added some data on the composition of blood in relation to the regulation of neutrality.

The present work was undertaken to determine (1) the variations in the composition of the blood of normal cattle and (2) the magnitude of these variations taking place in 24 hours. The components studied were inorganic phosphorus, chlorine, carbon dioxide, and calcium. Some determinations were also made of potassium and magnesium.

### *Methods.*

The general scheme followed was that of Briggs (7). At the outset the making of all of the determinations made by him was contemplated. Later all but the four mentioned above were dropped.

The blood was collected from the jugular vein without stasis under oil in a tube containing lithium citrate and was immediately centrifuged. The plasma was used for analysis. Carbon dioxide was determined on the plasma by the constant pressure method of Van Slyke (8); chlorides, likewise on the plasma, by the method devised by the same author (9). For the calcium and phosphate estimations the proteins were precipitated by trichloroacetic acid in centrifuge tubes which were whirled for the 10 minutes required for the completion of the precipitation. The supernatant liquid was then poured through filter paper and the clear filtrate used for analysis. Inorganic phosphate was determined by Briggs' (10) modification of the Bell-Doisy method. Calcium was determined by measuring the sample (usually 15 cc. of filtrate corresponding to 3 cc. of plasma) into a small centrifuge tube, neutralizing to methyl red as recommended by Shohl (11), precipitating the calcium as oxalate, and titrating it in the same tube after centrifuging and washing. This procedure was compared with the original technique of Kramer and Tisdall (12) using diluted plasma instead of trichloroacetic acid filtrate and with the more recent colorimetric method of Briggs (13). A series of determinations was also run in which the plasma was ashed with sulfuric acid, taken up with hydrochloric acid, neutralized to methyl red, and estimated as above. The results by the four methods are given in Table I.

Briggs' technique was followed for the potassium (7) and magnesium (14) determinations.

*Composition of Normal Beef Blood.*

The results given below and summarized at the end of this paper represent analyses of 122 blood samples from about 40 animals of various ages and sexes. The youngest animal bled was about a year old. All were on ordinary herd rations except that calcium phosphate was added in certain instances as indicated.

The values plotted in Fig. 1 were obtained on 8 consecutive days on each of four animals. They show the extent to which the

TABLE I.  
*Comparison of Methods for Determination of Calcium.*

Sample No.	Method 1.*	Method 2.†	Method 3.‡	Method 4.§
1	11.1	10.6	10.9	10.7
2	10.2	10.1	10.2	10.4
3	11.2	10.8	11.4	11.1
4	10.2	10.2	10.1	10.0
5	10.1	10.7	9.9	10.6
6	10.2	11.1	10.0	10.7
7	10.7	10.5	10.6	10.6
8	10.0	10.9	10.9	10.3

\* Kramer and Tisdall's (12) method.

† Briggs' method (13).

‡ 25 cc. of plasma ashed.

§ Same as Method 1 except that trichloroacetic acid filtrate was used instead of plasma.

constituents may vary in 24 hours. It is of course granted that greater variations may take place.

DISCUSSION.

*Phosphorus.*—Abderhalden's results on inorganic phosphorus in blood serum were 8.47 and 6.20 mg. per 100 cc. (2.73 and 2.00 mm per liter). Ten determinations by Hart and his collaborators gave values ranging from 2.25 to 5.75 mg. per 100 cc. (0.73 to 1.85 mm per liter) and averaging 4.10 mg. (1.32 mm per liter). 58 determinations taken from the paper by Meigs, Blatherwick, and Cary ranged from 3.9 to 8.3 (1.25 to 2.67 mm



per liter) with an average of 5.4 mg. per 100 cc. (1.74 mm per liter). Our own range from 3.00 to 8.99 (0.97 to 2.90 mm per liter) with an average of 5.87 mg. per 100 cc. (1.89 mm per liter) for 122 estimations.

It is commonly held that inorganic phosphorus is one of the constituents of the blood that, in mature animals, is influenced

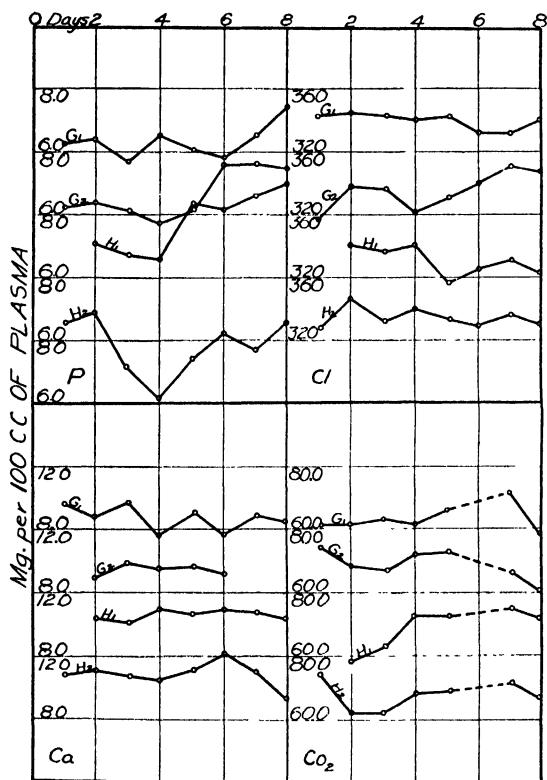


FIG. 1. Daily variation in composition of beef blood.

by the amount of phosphorus in the diet. Many of the data leading to this conclusion have been secured on animals other than cattle but Meigs, Blatherwick, and Cary (5) and Blatherwick (6) report results on cows supporting this assertion. We have two sets of experiments on three cows each which afford further confirmation of this. The results are shown graphically

in Figs. 2 and 3.<sup>1</sup> The curves especially in Fig. 2 show a uniform tendency to rise when calcium phosphate in the form of either bone meal or raw phosphate rock was added to the ration. The irregular results in Fig. 3 during the phosphate rock period are probably due to the difficulty encountered in getting the animals to eat the ration.

It should be noted that in no case was the phosphate content raised above that encountered under usual conditions of feeding. We have observed fluctuations in blood phosphate as wide as these with animals on a constant diet. Were it not for the fact

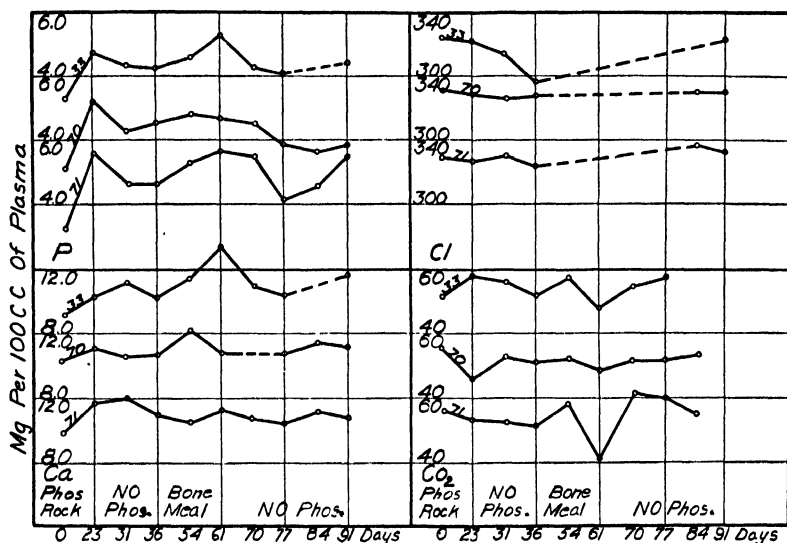


FIG. 2. Effect of ration on composition of beef blood.

that the rise in the curves took place simultaneously with the increase in phosphorus intake and that it was manifested in all the animals under observation, the results would be quite unconvincing. They would have been still more conclusive had there been daily samples with the formation of plateaux instead of peaks in the curves.

<sup>1</sup> These results form part of the theses submitted respectively by W. B. Jones and J. H. Mullen for the degree of Master of Science, Michigan State College.

Other causes of phosphate variation are still obscure and we have been able to correlate them with no other blood constituent nor in most cases with any definite cause. In Fig. 1 it will be observed that the phosphate curves are quite irregular, indicating variations as high as 1.87 mg. per 100 cc. of plasma (0.60 mm per liter) in 24 hours and 2.89 mg. (0.93 mm per liter) during the 8 days of the experiment. These animals were all on the same rations and were living under identical conditions.

*Chlorine*.—Abderhalden (1) found 369 mg. of chlorine per

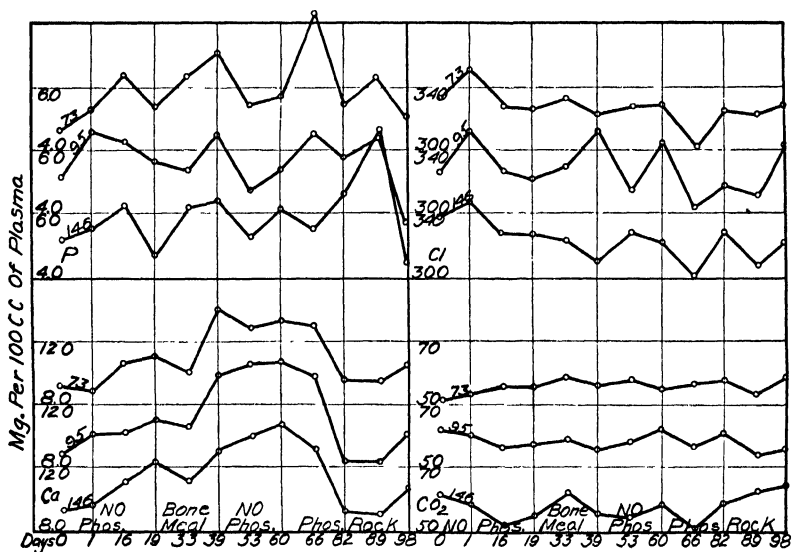


FIG. 3. Effect of ration on composition of beef blood.

100 gm. of serum (104.0 mm per liter) in the two samples that he analyzed. We are unaware of any extensive set of analyses of beef blood for chlorine. Our results show that this element is remarkably constant in amount, 110 analyses ranging from 294 to 357 (82.9 mm to 100.6 mm per liter) with an average of 329 mg. per 100 cc. (92.9 mm per liter). In the day to day experiment the greatest variation in any one 24 hour period was 24 mg., while the maximum fluctuation throughout the 8 days was 39 mg. The maximum and minimum values for the group were 318 and 357 mg. (89.9 mm and 100.6 mm per liter) respectively.

*Carbon Dioxide.*—Blatherwick (6) and also Hart (2) have reported studies on the carbon dioxide content of beef blood. Twenty-two determinations made by the former on sixteen cows showed a variation between 55.1 and 68.3 volume per cent (24.8 and 30.7 mm per liter) with an average of 61.5 per cent (27.7 mm per liter). He found a decided response in CO<sub>2</sub> capacity to changes in ration, acid-forming grain mixtures causing a decrease in CO<sub>2</sub> while base-forming food caused it to rise. He found no relation between CO<sub>2</sub> capacity and pregnancy.

112 determinations on our animals gave a range of 41.4 to 75.8 volume per cent (18.6 to 34.1 mm per liter) with an average of 59.2 (26.6 mm per liter). The maximum daily variation for a single animal was 14.0 per cent (6.3 mm per liter) while for the 8 days it was 17.8 per cent (8.0 mm per liter). The range for the four animals throughout the week was from 58.0 to 75.8 volume per cent (26.1 to 34.1 mm per liter).

The extreme values quoted above are, in our experience, rather unusual. We have observed that such figures occasionally occur for a short period or for only a single sample and presumably represent some temporary disturbance in the animal which is not otherwise manifest. The usual range of carbon dioxide content we find to be between 50 and 70 per cent (22.5 and 31.5 mm per liter).

*Calcium.*—Abderhalden (1) found 11.9 and 11.1 mg. (2.98 to 2.78 mm per liter) Ca in the samples of beef blood that he analyzed. Meigs, Blatherwick, and Cary (5) analyzed many samples of blood from cows in various stages of lactation and pregnancy. Hart (2, 3, 4) and Blatherwick (6) have also secured some results on this element.

Forty-one determinations from the paper of Meigs, Blatherwick, and Cary (5) gave an average of 9.72 mg. per 100 cc. (2.43 mm per liter) and ranged from 8.9 to 13.7 mg. (2.22 to 3.43 mm per liter). Blatherwick (6) reports 10.3, 11.3, and 10.4 mg. (2.58, 2.83, and 2.60 mm per liter) in three analyses. Hart (3) reports twelve determinations ranging from 8.79 to 24.42 (2.20 to 6.11 mm per liter) and averaging 16.25 mg. per 100 cc. (4.06 mm per liter) and nine (2) varying from 9.2 to 11.6 (2.30 to 2.90 mm per liter) and averaging 10.0 mg. per 100 cc. (2.50 mm per liter).

117 estimations in the course of our work averaged 11.0 mg.

(2.75 mm per liter) and ranged from 7.7 to 14.7 mg. per 100 cc. of plasma (1.93 to 3.68 mm per liter). We found a maximum 24 hour fluctuation of 2.0 mg. (0.5 mm per liter), while the greatest variation during the 8 days was 2.8 mg. (0.7 mm per liter).

The blood calcium in animals other than cattle has usually been considered to be constant irrespective of variations in the intake of this element except in cases where the blood calcium is abnormally low (15). Meigs, Blatherwick, and Cary (5) were unable to raise appreciably the blood calcium in cattle by feeding calcium chloride. Blatherwick (6) reports a higher value for this element in an animal when on a diet of alfalfa hay than when on a ration of grain or corn silage and ascribes this result to the greater quantity of calcium in the hay. He also reports a value of 12.1 mg. per 100 cc. of plasma for a cow on alfalfa hay. This being above a normal value of about 10 mg. is advanced as additional evidence that hay can raise the blood calcium. These results do not appear to be conclusive. The first one represents a single value about 1 mg. higher than the results obtained several days before and after it. We have had variations twice as great occur with an animal on a constant diet and all of the sets of observations reported above had this or greater variations during 24 hours at least once during the 8 days that the animals were under observation. The second case is likewise doubtful as values of 12 mg. are not unusual with no abnormally high intake of calcium. Hart and his collaborators even report marked decreases in blood calcium when animals were changed from timothy to alfalfa hay with an increased calcium intake. They also have published figures showing the independence of the calcium intake and the blood calcium.

Our own results, however, apparently indicate a tendency towards an increase in blood calcium with an increase in the calcium in the ration. Particularly in Fig. 2 there seems to be a consistent rise in the blood calcium during or immediately following the periods of high calcium intake. In Fig. 3 the bone meal apparently caused a marked increase in the blood calcium. However, the fact that this high level persisted over a 3 week period during which no calcium was added to the ration makes any conclusion drawn from the picture of doubtful value. The doubt is intensified by the decided drop during the period of feeding

phosphate rock, although, as mentioned above, the difficulty in feeding this material caused considerable disturbance in the animals. We are, however, inclined to believe that an increase in intake of either phosphorus or calcium causes a tendency towards an increase in the concentration of the corresponding element in the blood but the actual rise does not usually carry the values beyond the normal range and the picture may be obscured by changes produced by other factors more potent than the one under consideration.

The following case may be cited in this connection. We had under observation four calves which were suffering severely from depraved appetite. They were bled when first called to our attention on January 15. Bone meal and cod liver oil were added to their ration and they were bled again on the dates indicated. In two of these four calves the blood phosphate and in three of them the blood calcium was markedly increased. None of these animals was in normal state of health and the results must be considered with that point in mind. The results are shown in Table II.

*Potassium*.—Thirty-one estimations of potassium ranged from 16.4 to 41.3 (4.2 to 10.5 mm per liter) with an average of 27.3 mg. per 100 cc. of plasma (7.0 mm per liter).

*Magnesium*.—Twenty-six determinations gave results varying from 0.31 to 3.08 mg. per 100 cc. of plasma (0.13 to 1.26 mm per liter), average 2.16 (0.89 mm per liter).

#### SUMMARY.

An analysis of more than a hundred samples of blood from normal mature cattle showed the following values:

	Range.		Average.		Maximum 24 hr. variation.	
	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm
Inorganic phosphorus.....	3.00-8.99	0.97-2.90	5.87	1.89	1.87	0.60
Chlorine.....	294-357	82.9-100.6	329	92.9	24	0.68
Calcium.....	7.7-14.7	1.93-3.68	11.0	2.75	2.0	0.50
Carbon dioxide.....	41.4-75.8	18.6-34.1	59.2	26.6	14.0	6.3
Potassium.....	16.4-41.3	4.2-10.5	27.3	7.0		
Magnesium.....	0.31-3.08	0.13-1.26	2.16	0.89		

TABLE II.  
*Effect of Bone Meal and Cod Liver Oil on Ca and P in Blood of Calves.*

Animal No. . . .	1				2				3				4			
	P		Ca		P		Ca		P		Ca		P		Ca	
Date.	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm	mg. per 100 cc.	mm
Jan. 15	7.26	2.34	9.5	2.38	7.14	2.30	10.8	2.70	7.18	2.31	11.4	2.85	6.31	2.03	11.3	2.83
" 21									9.33	3.02	12.3	3.08				
" 25	8.68	2.80	12.5	3.12	6.94	2.24	13.0	3.25	8.33	2.68	12.6	3.15				
Feb. 11	8.12	2.62	12.7	3.18	7.10	2.29	13.0	3.25	7.35	2.37	12.1	3.03	5.89	1.90	11.6	2.90

Increased intake of calcium phosphate produced a slight increase in the inorganic phosphorus and calcium in the blood but the values were still within the normal range. Other factors produced greater changes in these two constituents.

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# **STUDIES ON THE CHEMICAL COMPOSITION OF BEEF BLOOD.**

## **II THE COMPOSITION OF THE BLOOD OF DAMS AND CALVES IMMEDIATELY AFTER CALVING.\***

By C. S. ROBINSON AND C. F. HUFFMAN.

WITH THE ASSISTANCE OF R. K. PADDOCK, K. L. BURT, AND G. E. TAYLOR.

*(From the Chemical Laboratory and Department of Dairy Husbandry of the Michigan Agricultural Experiment Station, East Lansing.)*

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The composition of the blood of mothers during pregnancy and immediately following delivery has been studied by a number of investigators most of whom have used women as subjects.

De Wesselow (1) found that the inorganic phosphorus in the maternal blood fell during the later months of pregnancy but rose to abnormally high levels during lactation. Exceptions to the rule were not infrequent. Hess and Matzner (2) found the inorganic phosphorus of the blood of pregnant women to be approximately normal. At birth it was almost invariably lower than that of the infant. The inorganic phosphorus in infant blood was lower at birth than at 1 month of age or older. Riesenfeld, Rose, and Handelsmann (3) found the inorganic phosphorus in maternal blood to be lower than that of the infant. Meigs, Blatherwick, and Cary (4) found that the inorganic phosphorus in the blood of cows diminished towards the end of pregnancy. In calf blood it was fairly high at birth and increased somewhat to a maximum at about 6 months of age. Hasselbalch and Gammeltoft (5), in a comprehensive study of the mechanism of neutrality regulation in pregnant women, demonstrated by a study of the hydron concentration in relation to the  $\text{CO}_2$  tension, the existence of a condition of acidosis. Losee and Van Slyke (6) found in toxemic pregnancy a mild to moderate acidosis, but at most a slightly subnormal plasma bicarbonate in normal pregnancy. Marrack and Boone (7) found a lowered  $\text{CO}_2$  tension which produced "a moderate alkalemia and reduction of plasma bicarbonate." In the work of Rowe (8) "evidence of a marked lowering of the tension of alveolar carbon dioxide in normal pregnancy is presented com-

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parable to that observed in well defined ketosis." Marriott (9) concluded that a condition of acidosis existed in infants. Schloss and Harrington (10) found that the plasma bicarbonate in normal infants was lower than in adults. Seham (11), however, was unable "to establish a lower  $\text{CO}_2$  tension which is indicative of the so-called 'acidotic state'" and said that "results with the alkali tolerance test for normal new-borns do not indicate an acidosis." Cook (12) found a reduction of plasma bicarbonate in pregnancy. Blatherwick (13) found no evidence of acidosis in pregnant cows. The blood bicarbonate of calves was higher than that of cows. Bell (14) advanced the idea that calcium plays an all important rôle in the genital functions and suggests that parturition takes place when the fetus ceases to absorb calcium which accumulates in the system causing contraction of the uterine muscles. Lamers (15), while demonstrating the inaccuracy of Bell's analytical methods, apparently confirmed his contention that the blood calcium is higher in women in the later months of pregnancy than in non-pregnant women and higher in labor than in the preceding months. He called attention, however, to the wide individual variations. His conclusions were based on averages. Kramer, Tisdall, and Howland (16) studied the blood calcium of a large number of normal children. They found the concentration to be remarkably constant. In two abnormal conditions, tetany and renal insufficiency, the values were low but under no circumstances were they raised to an unusual extent. The figures for children were apparently slightly higher than for adults. De Wesselow (1) found a diminution in calcium in pregnancy but observed that the figures remained within the physiological range. He concluded that, "The demand for calcium for the foetal needs does not therefore lead to any definite depletion of the maternal blood." After delivery the calcium returned rapidly to normal again. Hess and Matzner (2) found that the maternal blood calcium was usually lower than the calcium in the blood of the child. Widdows (17) in a study of blood calcium during pregnancy found a definite decrease in this element with a rise after parturition. She called attention to the unreliability of averages and found it necessary to follow individual cases through. Cook (12) found "no considerable deviation from normal" in the blood calcium. Hellmuth (18) found the fetal serum to be always richer in calcium than the serum of the mother. Meigs, Blatherwick, and Cary (4) obtained results which indicated if anything that the blood calcium in pregnant cows was lower than in farrow heifers. New born heifers showed unusually high values which, however, decreased during the early months of life. They discussed these results at length and suggested a connection between the high calcium of new born calves, of infants, and pregnant mothers and the acidotic conditions found to exist. This suggestion was later discredited by Blatherwick (13) who found no consistent relation between plasma bicarbonate and calcium. He found a higher level of blood calcium in calves than in cows.

Summing up the results to date it may be concluded that the inorganic phosphorous in the blood of women is low in the later

months of pregnancy, rising after delivery perhaps to unusually high levels. It is lower than the blood of the infant at birth. This element is low in the blood of infants but rises during the 1st month. In cows it is likewise lowered in pregnancy and in calves it is lower at birth than at 6 months of age.

In mothers and their offspring a condition of acidosis exists resulting in a lowered bicarbonate. In cows this has not been demonstrated and apparently in calves an unusually high blood bicarbonate is found.

The results on blood calcium are conflicting, some investigators reporting an increase and some a decrease in this element in pregnancy. Apparently the calcium in the blood of children is higher than it is in maternal blood. The same condition appears to exist in cows and calves.

The present work was undertaken with the view of confirming and extending preceding observations for comparison with conditions resulting from the use of certain restricted rations for calves.

Four blood constituents were studied, inorganic phosphorus, chlorine, carbon dioxide, and calcium. Briggs' modification (19) of the Bell-Doisy method was used for the determination of the phosphate; Van Slyke's methods were used for the carbon dioxide (20) and chlorine (21) estimations; and the modified Kramer-Tisdall method for the calcium (22).

In connection with this last method it was observed that the trichloroacetic acid precipitate in the blood of new born calves was in the form of such a fine colloidal suspension that it was not thrown down by centrifuging at speeds up to about 3500 R.P.M. nor was it held back by filter paper. Increasing the concentration of the trichloroacetic acid helped some but did not entirely obviate the difficulty. The results of the calcium and phosphate determinations are both affected by this behavior unless care is taken to prevent it. The colors of the solutions used for the former procedure are so altered by the suspended solid that accurate comparison with the standard is impossible, while the organic matter carried down with the oxalate precipitate in the latter method reacts with the permanganate yielding high results. We avoided the difficulty by allowing the tube containing the trichloroacetic acid precipitate to stand overnight. This usually resulted in a

clear layer of supernatant liquid from which samples could be pipetted.

The blood samples were collected from the jugular veins without stasis, allowed to run into centrifuge tubes containing lithium citrate under a layer of neutral paraffin oil, and centrifuged at

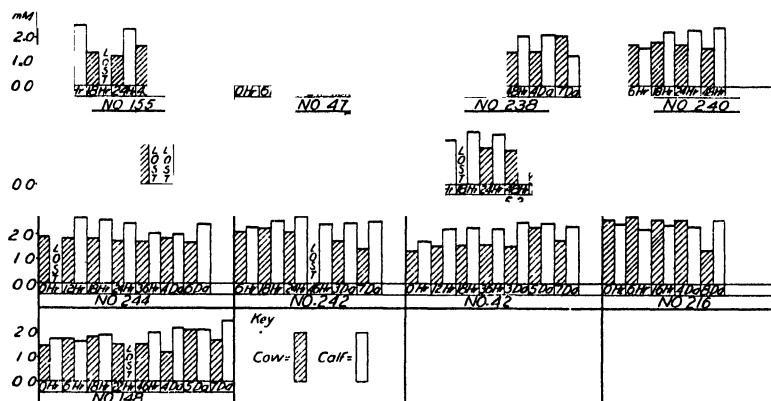


FIG. 1. Inorganic phosphorus content of blood of cows and calves.

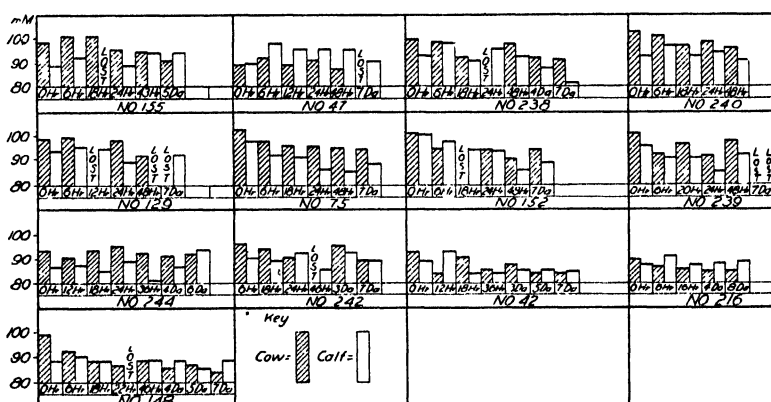


FIG. 2. Chlorine content of blood of cows and calves.

once. The first samples were taken as soon after calving as possible, usually within 15 minutes. The attempt was made to get samples at 6, 12, 18, 24, 48 hour periods unless the interval came during the night, in which case the sample was omitted. After the 48 hour sample a regular schedule was not followed but samples

were taken at intervals for several days as indicated. The data are shown in Figs. 1 to 4.

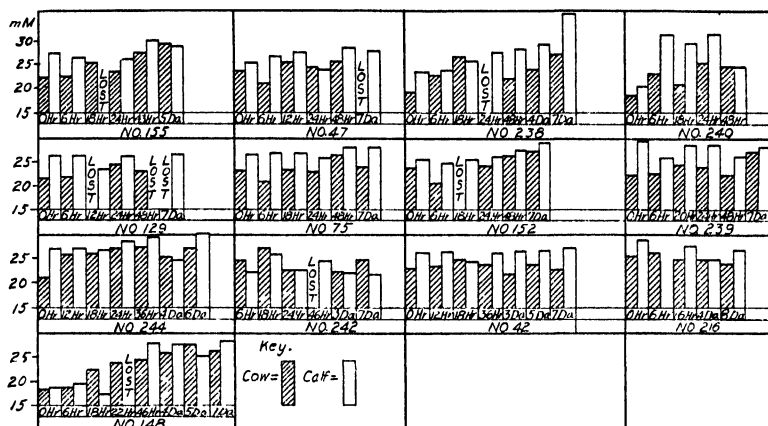


FIG. 3. Carbon dioxide content of blood of cows and calves

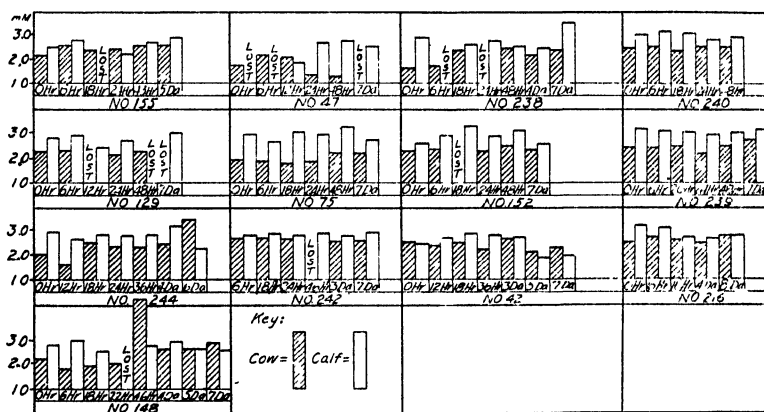


FIG. 4. Calcium content of blood of cows and calves.

#### DISCUSSION.

*Inorganic Phosphorus.*—In all but two cases the inorganic phosphorus content of the maternal blood was below that of the calf at birth. The figures varied from 0.90 to 2.60 millimols per liter with an average of 1.70 which is well within the range for normal cows. In fact, only one value was without this range. The values

for the calves were approximately 0.64 mm higher but still within the same limits. They varied from 1.19 to 2.71 and averaged 2.23 millimols per liter of plasma. The values for maternal inorganic phosphorous usually rose during 6 to 18 hours after calving to a rather definite maximum which was followed by a depression. Beyond this the fluctuations were irregular and present a picture differing in no way from that observed in normal cattle, although in most cases the final values were higher than the initial ones. It is apparent, however, that they held to the lower levels of the normal range, only 35 per cent of them attaining a value of over 1.89 mm, the normal average. Whenever this figure was exceeded either at the time of calving or later, it seemed to be followed by a prompt return to the lower level. In only one case was the figure greater than this a week after calving.

In the calves the blood phosphorus was quite consistently higher throughout the week than the maternal blood phosphorus and the great majority of cases showed higher values at the end of the week than at birth. At the end of 5 to 8 days the average was 2.37 mm.

These results agree in general with the findings of previous investigators both on women and cows except that the maximum for calf blood is probably reached much sooner than 6 months as believed by Meigs, Blatherwick, and Cary (4). Some of our results which will be published later indicate that a definite maximum is frequently attained during the 2nd month. De Wes-selow's (1) statement that during lactation the inorganic phosphorus in maternal blood reaches abnormally high levels does not hold true for cows, at least during the 1st week, although our results might have presented a different picture at a later date when lactation had reached a maximum.

*Chlorine.*—In all cases observed, the chlorine content of the maternal plasma was relatively high at the time of calving. It varied from 88.8 to 102.1 and averaged 97.4 millimols per liter. These figures are slightly higher than those found for normal cows. The fact that in all but three cases there was an immediate drop in chlorine after birth, followed by a steady decline throughout the period of observation substantiates the idea that the chlorine content of the maternal blood was unusually high at calving. At

the end of 5 to 8 days the values ranged from 84.0 to 94.2 and averaged 89.3 mm.

In only one case was the maternal blood poorer in chlorine than the calf blood at birth. The latter varied from 86.6 to 100.4 and averaged 91.9 millimols. There was a pronounced tendency for the values to rise slightly during the first 6 hours after which they diminished. The average decrease during the period of observation was much lower in the calves than in the cows. It may be concluded that in this respect calf blood is more nearly normal at the time of delivery than is that of the mother.

*Blood Bicarbonate.*—The blood bicarbonate in cows at calving varied from 18.4 to 25.2 millimols per liter and averaged 21.8. The rapidity with which the values returned to normal varied greatly in different animals. As has been shown elsewhere (22), values below 24.8 millimols are not unusual in apparently normal cows, such figures constituting about 30 per cent of the cases studied. If this is selected as the low limit of normality it will be observed that in only about 50 per cent of the cases has the blood bicarbonate permanently returned to the normal range after 48 hours. In four of the fourteen animals there was either a slight diminution or only an insignificant gain during the week of observation. On the whole though, there was a very evident tendency on the part of the values to rise during this time. The range after 5 to 8 days was from 22.5 to 29.3 mm with an average of 25.8.

The blood bicarbonate of the mothers was invariably lower than that of their calves at birth. This relation held almost without exception throughout the whole period of observation. The only marked deviation from the rule was in the case of Cow 242. The values for this calf were unusually low and remained almost constant at a 22.5 millimol level for several weeks. It died at the age of 41 days of general septicemia.

The bicarbonate picture for the calves presents nothing very unusual. At birth the values ranged from 17.6 to 29.3 with an average of 24.2 millimols. There was a general though not universal trend upwards and values below the low limits for mature cattle were infrequent. At 5 to 8 days the range was 21.3 to 36.3 with an average of 28.1 mm.



Our results agree with those of a majority of investigators who have studied the problem with human subjects, both as regards the maternal blood itself and the relation between it and the blood of the offspring. Since we have no data on the composition of blood before calving, any conclusions as to the effect of pregnancy on this constituent are impossible. That a condition of lowered blood bicarbonate exists in both mother and young immediately after birth and for a period of several days thereafter is, however, apparent. In some cases the minimum was not reached for several hours after calving.

*Calcium.*—The calcium in cow blood at the time of calving varied from 1.68 to 2.55 and averaged 2.19 millimols per liter. With the exception of two low values all were within the physiological range for normal cows. In nearly all cases the sample at 6 hours was somewhat richer in calcium than the first sample taken and a general tendency for the figures to increase was marked. At 5 to 8 days the values ranged from 2.15 to 3.43 and averaged 2.61 mm.

The blood of the mother at calving time was lower in calcium than that of the calf. The latter varied from 2.45 to 3.17 and averaged 2.83 millimols per liter. Only three were below the average of 2.75 mm found for normal cattle though all were within the normal range. The tendency was for the figure to decrease during the 1st week rather than the reverse but this was not very pronounced. The average at the end of 5 to 8 days was 2.79 mm.

Our results are quite at variance with the contention of Bell (14) and the findings of Lamers (15) in women that the blood calcium is high at the termination of pregnancy. They tend rather to confirm the statements of other investigators that labor is accompanied by a drop in calcium. Calving is followed by an immediate rise in the concentration of this element, a change which is, however, frequently not completed for several days.

A survey of our results indicates that, in so far as the blood picture shows, the process of calving differs in no way from childbirth in women. It seems that the mother suffers the greater disturbance in the event since her blood is further from normal than is that of the calf.

## SUMMARY.

The changes in inorganic phosphorus, chloride, carbon dioxide, and calcium in the blood of cows and calves during the week following calving have been studied. The following observations were recorded:

1. In but few cases were figures found outside of the range for normal cattle but certain characteristic changes were noted.

2. The inorganic phosphate of the blood of cows was approximately 0.64 mm lower than that in the calf blood at birth. The values for cow blood rose slightly during the first few hours, and at the end of the week they were ordinarily higher than at calving time. This was likewise true for the blood of the calves.

3. The chlorine content of the blood of cows was always high at calving but dropped steadily during the period of observation. The calf blood was lower in chlorine than that of the mothers, rose slightly during the first few hours after birth, and then dropped, though not so consistently as that of the cows.

4. A reduced bicarbonate content in the blood of both cows and calves was apparent at birth. Occasionally it fell still lower during the first 24 hours. The value for the mother was lower than that of her calf and this relationship was not reversed during the 1st week although there was a tendency for both to rise.

5. The calcium in cow blood at calving is lower than that of the calf. The former showed a tendency to increase during the week following while the reverse was true for calf blood.

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## A NEW SULFUR-CONTAINING COMPOUND (THIASINE) IN THE BLOOD.

By STANLEY R. BENEDICT, ELEANOR B. NEWTON, AND  
JEANETTE ALLEN BEHRE.

WITH THE COOPERATION OF ALICE ROHDE DAVIS.

*(From the Department of Chemistry, Cornell University Medical College,  
New York City.)*

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In a previous paper one of us (1) reported the isolation in crystalline form of a compound responsible for the interference in the direct methods for the determination of uric acid in blood. Details were not given beyond pointing out that the compound is precipitated by silver lactate in presence of lactic acid, and is not freed from its silver combination as precipitated from tungstic acid blood filtrates, by treatment with the acid sodium chloride used in the Folin-Wu method for uric acid determination. It is the purpose of the present paper to describe the method of preparation, composition, and some of the properties of the new compound. We shall also report in a preliminary way upon the occurrence of the compound in human blood. We wish, however, to first point out that at the suggestion of one of us the compound which we shall describe was isolated in crystalline form from pig's blood and analyzed in this laboratory more than 5 years ago by Helen Davis Dugdale. Mrs. Dugdale determined the melting point and ultimate composition of the compound, and the figures then recorded in her note-books show beyond question that she had prepared the substance in pure form. Mrs. Dugdale left the laboratory before she had established the fact that the method of isolation she employed could be uniformly depended upon to yield a compound of definite purity. Hence we hesitated to publish her figures at that time. It is a pleasure to record here that Mrs. Dugdale's long and careful work had resulted in securing the compound sought in pure form and in quantities sufficient for complete elementary analyses.

The present work was the outcome of the observation by one of us (2) that an interfering uric acid-reacting compound is habitually precipitated by the silver lactate in the Folin-Wu method for uric acid determination in blood, but that this compound is not liberated by treatment with acid sodium chloride solution as is uric acid. Pig's blood was found to be especially rich in the compound, and being practically free from uric acid, either free or combined, was selected as the most promising source of the new material. Our first procedures for isolation of the compound were based upon treatment of the blood with tungstic acid, followed by precipitation of the filtrate with silver lactate. The silver precipitate (after washing with acid sodium chloride) was treated with hydrogen sulfide, upon the assumption that this would set free the remaining compound or compounds in combination with the silver. In presence of the tungstate such liberation is very slight, and it was this fact which led to the slow initial progress in the present work. Subsequently we found that extraction of the silver salt with boiling dilute hydrochloric acid would liberate the greater portion of the desired compound, and we prepared a considerable quantity of the material using this procedure as a basis. Still later we found that some samples of tungstate will remove all or nearly all of the desired compound from the blood during precipitation, while others show no such action. This finding led us to abandon preliminary precipitation of the blood by means of tungstic acid, and we have finally adopted the following procedure for the preparation of the compound.

5 liters of blood are usually handled at one time, the coagulation being carried out in large porcelain-enameled pails. The fresh, defibrinated blood is poured into five times its volume of boiling 0.01 N acetic acid, and the mixture boiled for about 1 minute and then poured on to a large fluted filter paper (S. and S. No. 595). The filtrate is boiled down to about twice the volume of the blood used, again filtered, and the filtrate cooled, preferably in an ice box. The last traces of protein are removed from this cold solution by treatment with 0.1 volume of colloidal iron (Merck's 5 per cent), and filtration. This filtrate is now placed in tall form cylinders or jars and treated with 0.2 its volume of silver lactate solution and 0.02 its volume of 10 per cent sodium tungstate solu-

tion.<sup>1</sup> The mixture is stirred and set in a dark place for the silver salts to settle. The supernatant fluid is then syphoned off and the suspended precipitate treated with 10 per cent sodium chloride solution in 0.1 N hydrochloric acid, using about 0.1 the volume of original blood used, and the precipitate again allowed to settle. This treatment removes some impurities, but does not appreciably decompose the silver compound of the new substance. After the precipitate has settled (30 to 40 minutes) the supernatant fluid is syphoned off and the precipitate washed into 50 cc. centrifuge tubes and washed with water by stirring and centrifugation until the test for chlorides is faint or negative. The precipitate is now washed into a casserole containing hot 0.5 N hydrochloric acid in amount equal to about 0.25 volume of blood used, and the mixture boiled (with stirring) for 3 to 5 minutes. This treatment sets free about 75 per cent of the desired compound from its silver combination. Subsequent extractions will remove additional amounts, but for practical purposes it does not pay to work up the dilute solutions thus obtained. The hydrochloric acid solution is allowed to stand overnight in an ice box, and is then treated with granulated zinc and a bit of platinum and allowed to stand for about 20 minutes. This treatment is not essential and has no effect upon the compound itself. It apparently removes some traces of silver and tungsten from the solution and seems to aid in securing better flocculation of the mercury salt which is next prepared.

After treatment with the zinc the mixture is filtered and the filtrate treated with dry mercuric acetate to make a 10 per cent solution of this salt. The mixture is shaken for a few moments to dissolve the mercuric acetate. A heavy white flocculent precipitate forms immediately, which is a double compound of the desired substance with mercuric chloride.<sup>2</sup> After 20 to 30 minutes standing the supernatant fluid is decanted and the precipitate

<sup>1</sup> The Folin-Wu silver lactate solution in 5 per cent lactic acid is used, prepared according to Benedict, S. R., *J. Biol. Chem.*, 1922, li, 195. The sodium tungstate solution is added to secure better flocculation of the precipitate.

<sup>2</sup> The new compound forms no insoluble compound with mercuric acetate. In the present case mercuric chloride is formed by interaction of the acetate and the hydrochloric acid already present in the solution.

centrifuged in 50 cc. tubes. It is washed twice by thorough stirring with 1.5 per cent mercuric chloride solution, and finally once with 95 per cent alcohol containing about 0.01 per cent hydrochloric acid. The mercury salt is next suspended in a moderate amount of water (about 200 to 300 cc. for the precipitate representing 5 liters of blood) and decomposed with hydrogen sulfide. This decomposition requires about 20 minutes with frequent shaking or stirring during the entire time. After the precipitate is well darkened the mixture should be heated to boiling and the passage of hydrogen sulfide continued for about 10 minutes. After stopping the hydrogen sulfide the excess of this gas is removed by vigorous boiling for 5 minutes, and the mercury sulfide removed by filtration. The residue on the filter is washed with successive portions of boiling water and the combined filtrate concentrated to a small volume by boiling. The solution is then transferred to a crystallizing dish and concentrated on the water bath to a volume of about 2 cc. The dish is then placed in an ice box, preferably at a temperature only 2 to 3 degrees above 0. The cooling is very essential. The new compound usually begins to crystallize within an hour or two, and crystallization is complete in from 12 to 24 hours. Large, slightly colored prismatic crystals are thus obtained, which analysis shows to be the hydrochloride of the new compound. The yield averages from 300 to 400 mg. from 5 liters of blood, though we have obtained as high as 550 mg. from this amount of blood.

The hydrochloride of the new compound thus obtained may be recrystallized from water and thus obtained in pure form. The free compound is prepared by crystallization of the hydrochloride as first obtained, from pyridine, as follows. Dissolve 1 gm. of the compound in 5 cc. of water and add pyridine in small amounts with stirring until about 15 to 20 cc. have been added. Set in an ice box for 24 hours. The compound should separate in snow-white prismatic needles. Filter on hardened paper with suction and wash with successive portions of pyridine. Dry in an air bath at 100 to 110° for some hours, or leave exposed to the air after preliminary drying, until all traces of pyridine have been removed.

We have prepared about 10 gm. of the new compound and have studied its composition and some of its properties, but have not

yet succeeded in elucidating its molecular structure. In view of the fact that the compound occurs in human blood in surprisingly large amount as we shall show later, and that it can be easily quantitatively determined, we feel that a name for it as a convenient means of reference is desirable. The sulfur content of the compound differentiates it sharply from other non-protein organic constituents of the blood thus far isolated, and for this reason we have selected the name thiasine<sup>3</sup> for the new compound.

Thiasine melts sharply at 262–263. Qualitative tests showed the presence of C, N, and S, and the absence of P and the halogens. Elementary analyses yielded the following results.<sup>4</sup>

	Found.	Calculated for $C_{13}H_{10}N_4O_5S$ .
Carbon.....	47.35	48.0
Nitrogen.....	18.20	18.6
Hydrogen.....	6.55	6.6
Oxygen.....	17.10	17.2
Sulfur.....	10.80	10.6

Molecular weight determinations have not been completed, but calculation on the basis of the hydrochloric acid content of the hydrochloride leave no doubt that the molecular weight agrees with that demanded by the above formula, *viz.* 300. Such a molecular weight would require a hydrochloric acid content of 10.8 per cent for the monohydrochloride. Our figures range from 10.9 to 11.8 per cent hydrochloric acid in thiasine hydrochloride.

Thiasine contains no  $\alpha$ -amino acid nitrogen (Van Slyke's method). It is very stable toward boiling 20 per cent hydrochloric acid and toward strong ammonia, as indicated by no change in its color-yielding power in the uric acid reaction after such treatment. Alkaline copper solutions do not yield a precipitate of cuprous oxide when boiled in presence of thiasine. Strong solutions of thiasine, when treated with saturated picric acid solution yield an amorphous flocculent precipitate. This precipitate dissolves readily upon warming, and separates again upon cooling. Solutions of thiasine when treated with picric acid and

<sup>3</sup> Thiosinamine is a well known compound, but we have failed to find any record of the previous use of the name thiasine.

<sup>4</sup> We are indebted to Mr. Emil Osterberg for making the elementary analyses.



sodium hydroxide as in the determination of creatinine in blood yield no more color than a blank under similar conditions, even with 36 hours standing. This statement holds for solutions at least ten times as concentrated in thiasine as blood filtrates may be. With very strong solutions of thiasine a trace of color just distinguishable from a blank may develop after several hours. The reaction with alkaline pictrate solutions can, however, be considered entirely negative for practical purposes. Thiasine cannot be a contributing factor in development of the color in the creatinine reaction given by blood filtrates. Thiasine yields no color with nitroprusside and sodium hydroxide. Subsequent addition of acetic acid yields a faint coloration, greenish in tinge. Millon's reaction is negative. Thiasine hydrochloride solutions behave in peculiar fashion toward silver nitrate solutions. Addition of silver nitrate to moderately dilute solutions of thiasine hydrochloride causes only a faint opalescence, and no precipitate forms. Addition of acetic acid causes no change, but upon addition of strong nitric acid a precipitate forms at once, which from strong solutions contains most of the thiasine as well as the hydrochloric acid. On treatment with water after centrifuging, this precipitate will dissolve, forming a thick opalescent solution. Thiasine cannot itself dissolve added silver chloride, so that the results just cited indicate that the thiasine holds hydrochloric acid in some non-ionizable form. The point is of interest in connection with the question of chloride transportation in the organism and we contemplate a more thorough study of this question. It may be noted here that our chlorine determinations upon thiasine hydrochloride have been made by distillation in a glass apparatus from sulfuric acid and potassium permanganate, the distillate being received in an excess of potassium iodide solution and the liberated iodine titrated with thiosulfate.

Thiasine is strongly dextrorotatory,  $[\alpha]_D = +116^\circ$ . As will be seen later in our discussion, thiasine occurs in human bloods in sufficient quantity to account for a considerable fraction of the rotation observed in filtrates from whole blood.

The only positive reaction which we have so far found to be given by thiasine is the reduction of the complex tungstic acid reagents commonly used for the determination of uric acid. In the presence of cyanide as the only alkali, and using the reagent

and technique described by one of us (2), 1 part of uric acid yields a color equal to that given by 7.5 parts of thiasine. Mixtures of uric acid and thiasine in any proportion yield a color equal to the sum of the two when determined separately, by the above technique. This shows that in blood filtrates there is no lessening of the interference due to thiasine on account of the presence of uric acid. This is quite contrary to what we expected, and we have checked the point very carefully a great number of times. Using carbonate as the alkali, as in the older uric acid methods, thiasine yields 4.6 times as much color in proportion to that given by uric acid as it does when cyanide is used as the sole alkali. Thus when carbonate is used as the alkali 1 part of uric acid yields as much color as 1.6 parts of thiasine. Using 0.5 cc. of reagent and 2 cc. of 2 per cent sodium hydroxide solution with 5 cc. of thiasine or uric acid solution, and no cyanide, uric acid yields only 77 per cent as much color as does an equal weight of thiasine.

There is one further point to be discussed before we take up the question of the occurrence of thiasine in the blood. This concerns the possibility that the sulfur found in thiasine has been introduced through the use of hydrogen sulfide to decompose the mercury compound. It is perhaps unfortunate that we have so far been unable to prepare thiasine from blood without the use of hydrogen sulfide at some stage, but we feel that there is indirect evidence which is irrefutable that we have not introduced the sulfur which is present in the purified compound. Thus we find that the hydrochloric acid extract of the silver lactate precipitate of the compound has exactly the same color-yielding power before and after treatment with hydrogen sulfide.

#### *The Occurrence of Thiasine in Blood.*

We have so far examined only pig and human blood for thiasine. In both of these bloods the thiasine is contained wholly in the corpuscles. This result was to be expected in view of the work of Guillaumin (3) and of Bulmer, Eagles, and Hunter (4). According to colorimetric determinations made directly upon the filtrate from pig's blood the thiasine content of this blood averages about 25 to 30 mg. per 100 cc. It is very probable that this figure is approximately correct, for in spite of the high solubility of thiasine hydrochloride we have actually isolated more than 10 mg. of

thiasine per 100 cc. of pig's blood in pure crystalline form. And we have obtained more than double this yield of thiasine in the form of the crude mercuric chloride salt. The thiasine content of pig and human bloods remains practically or wholly unaltered through some days of standing, providing putrefaction or hemolysis has not occurred.

Theoretically the determination of thiasine in human blood is very simple. Precipitation of the whole blood filtrate with silver lactate, followed by decomposition of the precipitate with acid sodium chloride solution as in the Folin-Wu method for uric acid should, and apparently does, effect a sharp separation of uric acid and of thiasine from other color-yielding substances which may be present in the blood. The uric acid fraction is determined as usual in the sodium chloride extract, while the silver residue remaining is used for determination of thiasine by dissolving in the cyanide used for completing the reaction, adding water and reagent, and heating as in the uric acid method. Using uric acid as a standard, the result is multiplied by the factor 7.5 to convert to thiasine.

We have determined the thiasine content of nearly 200 human bloods<sup>6</sup> by the procedure just outlined, and in addition have compared the figure obtained as the difference between the direct and indirect values for uric acid. This figure, we should expect, when multiplied by 7.5 should yield a figure for thiasine practically identical with that obtained when the silver residue after extraction with acid sodium chloride is employed, since, as we have stated earlier in this paper, thiasine and uric acid have full color-yielding value in presence of each other. In many bloods the figures for thiasine obtained by the two different procedures just outlined check almost exactly, while in many others the difference between the direct and indirect uric acid values is not so great as the result obtained when the thiasine is estimated in the purer form in the silver residue. We have studied the points involved here very carefully, and have concluded that some bloods contain material depressing somewhat the total color development in the filtrate directly. The method of choice for thiasine determination is therefore the separate determination of this substance and of uric acid as above outlined. Our results show a thiasine content for human blood averaging about 14 to 15 mg. per 100 cc. of blood,

<sup>6</sup> The bloods for this study were obtained through the kind cooperation of our colleague, Dr. Nellis B. Foster.

for a large group of unselected hospital and ambulatory cases. Our highest figures have been obtained in diabetic cases, many of which show 20 to 27 mg. of thiasine per 100 cc. of blood. This is not constant for diabetes however, though there seems to be no doubt of a tendency to high figures in this condition. In nephritis, on the contrary, the tendency is to normal or unusually low figures. This result might have been expected from the results of one of us (2) who found that the tendency to interference in the direct method for determination of uric acid in blood was not increased with increase of total non-protein nitrogen in the blood.

We are not presenting our figures for the thiasine content of human blood, or the method for the estimation of thiasine in detail at the present time for the following reasons. Tungstic acid is an uncertain precipitant for blood proteins when thiasine is to be determined, and we have so far found no means of telling whether a given sample of sodium tungstate can be used for the precipitation other than comparison of the filtrate obtained with the heat coagulation filtrate or with the filtrate from a sample of tungstate known to be satisfactory. We hope to have a solution of the problems involved here in the near future. Furthermore, the figures for thiasine in human blood have been so much higher than we expected, and have varied so independently of the total corpuscle content of the blood that we are inclined to exercise every possible precaution to prove that thiasine is the only compound involved before publishing our results in detail. We also feel that isolation of thiasine from human blood is a desirable prerequisite for an extended discussion of thiasine in human blood. We expect to accomplish such isolation in the near future.

We are continuing a study of the molecular structure of thiasine. We are also studying the distribution of the compound in the blood, urine, and tissues of various species.

*Addendum.*—Since the above paper was written an article by Hunter and Eagles has appeared (5) describing the isolation of a uric acid-reacting compound from pig's blood, to which the formula  $C_6H_{11}N_2O_3$  is assigned, and which is termed "substance X." The color value reported for "substance X" in terms of uric acid is identical with that of thiasine, but in practically every other reported finding the compounds seem to differ very materially. Even assuming an error in regard to the absence of sulfur and direction of rotation of "substance X," it is difficult to reconcile the reported findings for the elementary composition of this substance with those which would be yielded by a reasonably pure sample of thiasine.

In the present instance the publication by two different groups of workers upon the same general line of work at very nearly the same time may merit some words of comment.

In 1922 one of us reported (2) that all or most of the interfering substance in the uric acid method was precipitated by silver from the Folin-Wu filtrates, but was not liberated from this precipitate by treatment with acid sodium chloride solution, as is uric acid. This suggested to us a possible mode of procedure for isolation of the substance or substances involved, but we did not begin experimental work upon the question for some time. In January of 1924 one of us suggested to Mrs. Alice R. Davis that she take up this problem. Work along this line was soon begun by Mrs. Davis and one of us (E. N.). This work was carried out in the biochemical laboratory of the Harvard Medical School. Dr. Folin kindly offered the facilities of that laboratory for the work, but assumed no responsibility in regard to it. Up to the fall of 1924 thiasine had not been isolated, and work upon the problem was then begun in the laboratory at Cornell. In January, 1925, thiasine was isolated in pure crystalline form as the hydrochloride, and a little later, nitrogen, sulfur, and melting point determinations showed the identity of the compound with that previously isolated and analyzed in this laboratory by Mrs. Dugdale. In February, 1925, Bulmer, Eagles, and Hunter reported (4) that bloods contain a substance which interferes in the direct uric acid determination. These investigators apparently thought this substance "hitherto unrecognized," and termed it "substance X." Their only special chemical characterization of "substance X" was that it was not precipitated along with uric acid from blood filtrates by silver lactate, a statement which we had previously shown to be quite incorrect. Bulmer, Eagles, and Hunter made no mention of contemplating any additional work in relation to the interfering substance in blood.

In May, 1925, one of us published a communication (1) upon determination of uric acid in blood in which we reported that we had isolated the uric acid-reacting compound from blood, and that we should report promptly in more detail concerning it. It appears that some 2 months after this announcement, Hunter and Eagles submitted their paper upon the isolation of "substance X." If, in spite of the seeming irreconcilable differences, "substance X" should turn out to be identical with thiasine, we would point out that apparently thiasine was isolated and analyzed by us some time before Hunter and Eagles worked with "substance X," and that we have presented the first essentially correct description of thiasine and its properties.

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## FAT SOLVENTS.

By CHARLES E. BILLS.

*(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)*

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The literature affords but little information on the solubility of fats in organic liquids. Aside from the general belief that fats are soluble in hydrocarbons and esters, not much is known of their miscibility with more than a dozen substances. In view of the possibility of employing some of the less familiar solvents in biochemical investigations, 250 of these have been examined, and their miscibilities recorded in Tables I and II.

Most of the solvents were obtained from the Eastman Kodak Company, and they are said to be of a high degree of purity. The fats were good commercial samples of white coconut oil, unfrozen cod liver oil, raw linseed oil, refined olive oil, and filtered butter fat. The butter fat was specially prepared without the addition of artificial coloring.

The tests of solubility were made by mingling 1 ml. of the solvent and 1 ml. of the fat. It is probable that if different proportions were used a greater number of partially immiscible solvents could be tabulated. With equal volumes, some of the "immiscible" solvents exhibited partial miscibility, while others mixed only to a very small extent.

In the first series of experiments the 250 liquids were tested with cod liver oil. Twenty-eight of them were immiscible, and these were then tested with the other four fats. In many cases the other fats dissolved, so it appears that the term "fat solvent" cannot be applied with reference to all fats. The different natural fats vary greatly in solubility, and it is indeed possible to distinguish between them by this means. For example, pure butter fat can be distinguished from coconut margarine by its solubility in any of the following: acetic acid, allyl alcohol, chloroacetone, diacetone



TABLE I.  
*Solvents Immiscible with Various Fats; Their Color Reactions and Chemical Behavior.*

	Cod liver oil.	Butter fat.	Coconut fat.	Linseed oil.	Olive oil.
Acetic acid.	Immiscible.	Immiscible.	Miscible.	Immiscible.	Immiscible.
" anhydride.	"	"	Immiscible.	"	"
Aldol.	"	"	Miscible.	Miscible.	"
Allyl alcohol.	One sample im-	Miscible when	"	"	"
Chloroacetone.	miscible; an-	melted; sepa-			
	other sample	rates on cool-			
	miscible.	ing.			
Diacetin.	Immiscible.	Immiscible.	Immiscible.	Immiscible.	"
Diacetone alcohol.	"	"	Miscible.	Miscible.	"
Diacetyl.	"	"	Immiscible.	Immiscible.	"
Ethanolamine.	" Cry-	" Cry-	" Cry-	" Cry-	" Cry-
	tals develop in	tals develop in	tals develop in	tals develop in	tals develop in
	2 days.	2 days.	2 days.	2 days.	2 days.
Ethyl acetate.	Immiscible.	Immiscible.	Miscible.	Miscible.	Immiscible.
" alcohol.	"	"	Immiscible.	Immiscible.	"
Ethylene chlorohydrin.	" Olive-	" Flesh	Miscible. No	Miscible. Tran-	" Tran-
	green develops	color develops	color reaction.	sient but strong	sient pale blue
	in a few min-	in a few min-		blue-green	color develops
	utes, becoming	utes, changing		color develops	in a few min-
	dark brown in 2	to pale blue.		in a few min-	utes.
	hrs.			utes.	
" glycol.	Immiscible.	Immiscible.	Immiscible.	Immiscible.	Immiscible.
Ethyl lactate.	"	"	"	"	"

<b>Ethyl sulfate.</b>	Immiscible. Orange color develops at once, slowly blackening.	Immiscible. Pink color develops at once.	Immiscible. Faint color develops in 1 hr.	Immiscible. Green color develops at once, slowly blackening.	Immiscible. Pale brown color develops in 1 hr.
Formamide.	Immiscible.	Immiscible.	Immiscible.	Immiscible.	Immiscible.
Furfuraldehyde.	"	"	"	"	"
Furfuryl alcohol.	"	"	"	"	"
Glycerol.	"	"	"	"	"
Methyl alcohol.	"	"	"	"	"
" sulfate.*	A blue flash appears instantly, changing in a few seconds to intense purple, red, brown.	Pink color develops quickly, darkening to brown.	Pale reddish-orange color develops quickly.	Greenish-brown color develops quickly.	Orange color develops at once.
Monoacetin.	Immiscible.	Immiscible.	Immiscible.	Immiscible.	Immiscible.
Nitromethane.	"	"	"	"	"
Phenylhydrazine.	"	"	"	"	"
Isopropyl alcohol.	"	"	"	"	"
Pyridine.	One sample immiscible; another sample miscible.	Miscible.	Miscible.	Miscible.	Miscible.
Pyruvic acid.	Immiscible.	Immiscible.	Immiscible.	Immiscible.	Immiscible.
Triacetin.	"	"	"	"	"

\* Methyl sulfate. When this reagent was *shaken* with melted butter fat a purple flash appeared, quickly changing to yellow, red, and finally brown. Seal oil gave an orange color changing to brown; and herring and menhaden oils gave the same changes, but more rapidly. Sardine oil gave a color series similar to cod liver oil, but less rapidly. Dogfish liver oil gave immediately a red color changing to brown. Salmon oil gave immediately a reddish-brown color changing to brown. All these oils were immiscible.

TABLE II.

*Solvents Miscible with Cod Liver Oil; Their Color Reactions and Chemical Behavior.*

Acetal.	<i>n</i> -Butylamine.**	Diisobutylamine.
Acetaldehyde.	Isobutylamine.	Dichloroacetic acid.¶¶
Acetone.	Sec. butylamine.††	Dichlorobenzene (o- and <i>p</i> - mixture).
Acetophenone.	<i>n</i> -Butyl bromide.	Dichloroethylene.
Acetylacetone.	Isobutyl "	<i>s</i> -Dichloromethyl ether.***
Acetyl chloride.	Sec. butyl bromide.	Diethylamine.†††
Allylamine.*	Tert. " "	Diethyl carbinol.
Allyl bromide.	Isobutyl isobutyrate.	" ketone.
" ethyl ether.	<i>n</i> -Butyl chloride.	Dimethyl acetal.
" sulfide.	" chlorocarbo-	Dimethylaniline.
4-Amino- <i>m</i> -xylene.	nate.	Dimethyl- <i>n</i> -propyl carbinol.
Amino- <i>p</i> -xylene.	Isobutyl chlorocarbo-	Diphenyl ether.
Isoamylacetate.	nate.	Diphenylmethane.
<i>n</i> -Amyl alcohol.	Isobutylene bromide.	Di- <i>n</i> -propylamine.
Isoamyl alcohol (fermentation).	<i>n</i> -Butyl ether.	Diisopropylamine.
Tert. amyl alcohol.	" formate.	Dipropyl ketone.
<i>n</i> -Amylamine.†	Isobutyl "	Epichlorohydrin.
Isoamylamine.	<i>n</i> -Butylidene chloride.	Ethyl acetate.
Isoamyl chloride.	<i>n</i> -Butyl iodide.	Ethylal.
$\beta$ - <i>n</i> -Amylene.	Isobutyl "	Ethylbenzene.
Isoamyl formate.	Sec. butyl iodide.	Ethyl bromide.
" mercaptan.	<i>n</i> -Butyl nitrite.††	" <i>n</i> -butyl ether.
" nitrite.‡	" propionate.	" <i>n</i> -butyrate.
Aniline.	Isobutyl "	" carbonate.
Anisaldehyde.	<i>n</i> -Butyraldehyde.	" chloroacetate.
<i>o</i> -Anisidine.	Isobutyraldehyde.	" chlorocarbo-
Apiole.	Carbon tetrachloride.	nate.†††
Benzaldehyde.	Carvone.	Ethylene bromide.
Benzene.	Chloral.§§	" chloride.
Benzenesulfochloride.§	<i>o</i> -Chloroaniline.	" chlorobro-
Benzotrichloride.	Chlorobenzene.	mide.
Benzylamine.¶	$\beta$ -Chloroethyl acetate.	Ethyl ether.
Benzyl chloride.	Chloroform.	" ethylxanthate.
" ether.	Chloromethyl ether.	" formate.
" mercaptan.	<i>o</i> -Chlorophenol.	Ethylidene chloride.
Bromoform.	Chloropicrin.	Ethyl iodide.
$\alpha$ -Bromonaphthalene.	Cinnamaldehyde.	" malonate.
<i>n</i> -Butyl acetate.	Cresol ( <i>m</i> - and <i>p</i> -mixture).	" mercaptan.
Isobutyl "	Cyclohexene.	" nitrate.
Sec. butyl "	Diacetyl monomethox-	" ortho-formate.
<i>n</i> -Butyl alcohol.	ime.	
Isobutyl "	Dibenzylamine.	

TABLE II—Continued.

Ethyl oxalate.	Methyl formate.	Isopropyl bromide.
“ phthalate.	“ iodide.	<i>n</i> -Propyl <i>n</i> -butyrate.
“ propionate.	Methylphenylhydrazine.	Isopropyl chloride.
“ propyl ketone.	Methyl propionate.	<i>n</i> -Propyl chlorocarbonate.
“ sulfide.	“ <i>n</i> -propyl carbinol.	Isopropyl “
“ sulfite.	Methyl salicylate.	Propylene bromide.
“ trichloroacetate.	“ sulfide.	“ chloride.
“ <i>n</i> -valerate.	“ <i>n</i> -valerate.	<i>n</i> -Propyl ether.
“ isovalerate.	Nicotine.	Isopropyl “
Eugenol.	<i>o</i> -Nitroanisole.	<i>n</i> -Propyl formate.
Guaiacol.	Nitrobenzene.	Isopropyl “
<i>n</i> -Heptyl alcohol.	<i>n</i> -Octyl alcohol (primary).	<i>n</i> -Propyl iodide.
<i>n</i> -Hexane.	Oleic acid.	Isopropyl “
<i>n</i> -Hexyl alcohol.	Paraldehyde.	<i>n</i> -Propyl mercaptan.
Lead tetraethyl.	Pentachloroethane.	“ propionate.
Mesityl oxide.	Pentane (from petroleum).	“ sulfide.
Methyl acetate.	Petroleum ether.	Pyrrole.
Methylal.	<i>o</i> -Phenetidine.	Quinoline.
Methyl <i>n</i> -amyl ether.	Phenol (warmed).	Resorcinol dimethyl ether.
Methylaniline.	Phenyl acetate.	Salicylaldehyde.
Methyl anthranilate.	Phenylacetaldehyde.	Styrene. ¶ ¶ ¶
“ benzoate.	Phenylpropyl alcohol.	<i>s</i> -Tetrachloroethane.
“ <i>n</i> -butyl carbinol.	Phorone.	Tetrachloroethylene.
Methyl isobutyl ketone.	$\alpha$ -Picoline.	Thioacetic acid.
Methyl <i>n</i> -butyrate.	Piperidine.	Toluene.
“ <i>n</i> -caproate.	Propionaldehyde.	<i>o</i> -Toluidine.
“ carbonate.	Propionic acid.	Trichloroethylene.
“ chloroacetate.	<i>n</i> -Propyl acetate.	Triethylamine.
“ chlorocarbonate. §§§	Isopropyl “	Triethyl carbinol.
Methylcyclohexane.	<i>n</i> -Propyl alcohol.	Trimethylene chloride.
1-Methylcyclohexene.	<i>n</i> -Propylamine.	Trimethylethylene.
2-Methylcyclohexene.	Isopropylamine.	Isovaleraldehyde.
3-Methylcyclohexene.	<i>n</i> -Propylbenzene.	Xylene (mixture).
Methylene chloride.	<i>n</i> -Propyl bromide.	Xylidine “
Methylethyl ketone.		

\* On standing 4 weeks the tube was filled with a mass of fine crystals which melted at body temperature, and immediately reformed on chilling.

† Extensive production of fine crystals in 4 weeks; also considerable saponification.

‡ Solution became semisol'd red mass in 4 weeks. (Elaidin transformation?)

TABLE II—*Concluded.*

§ In a few minutes gave a flesh color, slowly changing to smoky-yellow and brownish-black.

|| In a few minutes gave a flesh color, slowly changing to pale orange and deep brown.

¶ Crystals developed in a few hours; in 1 week the tube was filled with a mass of fine crystals.

\*\* Slight production of crystals in 6 weeks.

†† Considerable saponification in 4 weeks indicated by froth on shaking with water.

‡‡ Solution became semisolid red mass in 4 weeks. (Elaidin transformation?)

§§ In a few hours gave a green color, slowly changing to red and brownish-black.

||| Instantly gave a reddish-purple flash, quickly changing to yellow, and later to brown, deep red, and black.

¶¶ Instantly gave a purple flash, quickly changing to brown and brownish black.

\*\*\* Instantly gave a blue flash, quickly changing to reddish-purple, and later to yellow, red, and brown.

††† In a few hours gave an orange color.

‡‡‡ In a few minutes gave a light red color, changing in several hours to light brown.

§§§ Gave same color changes as ethyl chlorocarbonate, but more rapidly. (Foot-note ‡‡‡.)

|||| Extensive production of fine crystals in 4 weeks.

¶¶¶ An excess of oil gave a clear viscous precipitate drying to a glassy mass, probably metastyrene.

alcohol, ethyl acetoacetate, ethylene chlorohydrin, or isopropyl alcohol. Undoubtedly, if all five fats had been tested with the 250 solvents, the list of immiscibles would be further increased.

It is evident, from Tables I and II, that closely allied substances may differ markedly in their solvent properties, so that it is difficult to predict by analogy the miscibility of a particular substance. Such differences are conspicuous between isopropyl alcohol and normal propyl alcohol, ethyl lactate and ethyl propionate, phenylhydrazine and methyl phenylhydrazine, ethyl sulfate and ethyl nitrate.

Several of the solvents reacted with the fats. The heretofore undescribed color reactions of cod liver oil are noteworthy, and the remarkable reaction of certain alkylamines in giving crystalline derivatives may be useful in the analytical separation of complex

fatty mixtures. When *n*-propylamine, *n*-butylamine, or *n*-amylamine is allowed to stand with cod liver oil for several weeks a mass of fine crystals slowly separates; the corresponding iso compounds do not yield the crystals. With allylamine the reaction is more pronounced, and with benzylamine it is the most rapid and extensive, the crystals appearing in a few hours at room temperature. All reactions are recorded in Table I or in footnotes to Table II.

Acknowledgment is made of the helpful suggestions received from Professor E. V. McCollum in the course of this work.



# A CRITICAL EXAMINATION OF FOUR METHODS COMMONLY USED FOR THE DETERMINATION OF SUGAR IN BLOOD.

By WALTER F. DUGGAN AND ERNEST L. SCOTT.

(From the Department of Physiology, Columbia University, New York.)

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The peculiar behavior of some of the well known blood sugar methods, when put to unusual uses, led us to the belief that it would be useful to add another to the already long series of critical studies of blood sugar methods.

It is a matter of common experience that, though a number of different methods may give concordant results with a given sample of blood, with a different blood the determinations may be quite at variance. This fact, that at times different methods agree while again they do not, is quite as interesting and probably more important than is the fact of their agreement, or lack of agreement, in itself. The methods which were examined were those of Benedict (1), Folin and Wu (4) as modified by Folin (5), Shaffer and Hartmann (11), and Hagedorn as described by Höst and Hatlehol (8).

Because of the great number of possible causes for variation between the results obtained by the different methods, it was thought advisable to begin the study with a comparison of the behavior of the several methods towards a solution of pure glucose in water. In this way it would be possible to determine the precision and accuracy of each method with the fewest possible complicating factors, and any discrepancies would be more easily interpreted.

## *I. Results of the Work with Solutions of Pure Glucose.*

### *A. Material and Apparatus.*

The three following samples of glucose were used.

1. A sample prepared from white commercial glucose in 1915. This sample was purified by several precipitations from methyl alcohol. Until



used, it was kept in a desiccator over calcium chloride. Its rotating and reducing powers agreed with those of pure anhydrous glucose.

2. A sample prepared by crystallization from acetic acid (Hudson and Dale (9)). The Exose of the Corn Products Company was the starting point and the sample, when purified, was kept in a desiccator, as was Sample 1. This sample was prepared about 9 months before the first of it was used. Like the first sample its rotating and reducing powers corresponded with those of anhydrous glucose.

3. In a few instances Exose was used as purchased. When so used it is indicated in the tables (Table IV). Its rotating power indicated that it was 91.1 per cent anhydrous glucose. All solutions of this sample were so made that a 1 per cent solution would have the rotating power of a 1 per cent solution of anhydrous glucose.

The polarimetric determinations were made on a Schmidt and Haensch two-field instrument sensitive to  $0.01^\circ$  of angular scale. Solutions of about 5 per cent were used for polarimetric work. A mercury arc lamp with a filter giving a wave-length of  $546 \times 10^{-8}$  mm. was used as the source of light. As noted in the descriptions of Samples 1 and 2 the specific rotation of glucose for light of this wave-length was, granting the purity of our preparations, found to agree with that given by Quisumbing and Thomas (10)  $[\alpha]_{546}^{25} = 62.02^\circ$ .

The anhydrous equivalent of the 5 per cent solution having been determined by rotation, a solution equivalent to 0.100 per cent anhydrous glucose was prepared from it by dilution. The reducing power of this diluted solution was then determined by the method of Quisumbing and Thomas (10). In all cases the agreement was within 0.3 per cent, whether the solutions were prepared from the purified samples or directly from Exose.

The colorimeter used was an ordinary Duboscq as made by Pellin. Readings were made by the use of the Chalet colorimeter lamp of Bausch and Lomb.

Inasmuch as we were primarily interested in the behavior of the several methods in blood sugar determinations, rather than in sugar methods in general, we have, in most instances, calculated our results to their respective blood sugar equivalents, instead of reporting the absolute indications of the sugar recovered. Thus when 0.20 mg. of sugar, absolute weight, is indicated by the Folin-Wu method it is reported as equivalent to a blood sugar of 100 mg. per 100 ml. The same absolute weight of sugar (0.20

mg.) by the method of Shaffer and Hartmann would indicate 40 mg. per 100 ml. of blood, by that of Benedict 31, and by that of Hagedorn 200. Or, again, in order to indicate 100 mg. per 100 ml. of blood there must be recovered by the method of:

	mg.
Folin and Wu.....	0.20
Shaffer and Hartmann.....	0.50
Benedict.....	0.64
Hagedorn.....	0.10

It will thus be seen that the requirements in sensitivity and precision of the different methods are quite varied. The difference in the significance of the absolute amounts of sugar recovered by the different methods depends upon differences in the amount of blood to which the volume of filtrate subjected to analysis corresponds. An aliquot part is used in all of the methods except that of Hagedorn. When it is desired to study the sensitivity of a method it becomes convenient to report the absolute weight of sugar indicated.

#### *B. Benedict's Modification of the Lewis-Benedict Method.*

With this method 2.0 ml. of blood, laked with 4.0 ml. of water, are treated with 19.0 ml. of a picric acid solution. The picric acid serves the double purpose of protein precipitant and oxidizing agent for the glucose. After the protein is precipitated, 8.0 ml. of the clear filtrate (equivalent to 0.64 ml. of the original blood) are treated with 1.0 ml. of 20 per cent sodium carbonate solution and placed in a boiling water bath for 10 minutes. At the same time, a tube containing 0.64 mg. of glucose in 4.0 ml. of solution, 4.0 ml. of the picric acid solution, and 1.0 ml. of the sodium carbonate solution is treated in like manner. After heating for the indicated time the tubes are cooled and the volume of each of the solutions made up to 12.5 ml. with distilled water. The blood sugar is then determined by comparing the amounts of picramic acid formed in the two tubes colorimetrically, it being assumed that under the conditions adopted this will vary directly as the sugar present. The reason for detailing the method in this way will become evident from what follows.

It is evidently assumed by the authors of the method that the

picric acid equivalent to that present in 6.5 ml. of the picrate-picric acid solution is removed with the protein precipitate, so that, after the precipitation is complete, the 8.0 ml. portion of the filtrate used for analysis contains the same amount of picric acid as does the 4.0 ml. added to the standard tube.

It is generally stated that this method gives fairly satisfactory results when used with normal bloods but that it is apt to give results which are too high, as compared with those obtained by other methods, when used with abnormal bloods, *e.g.* in diabetes. It may also give high results when used with bloods which may be presumed to have low protein content (Höst and Hatlehol (8)).

It may fairly be assumed that a reduced amount of protein in the blood will leave an excess of picric acid in the filtrate over and above that assumed by the author of the method to be present. Present knowledge does not permit us to write the equations for the reactions which occur in such a system but it would seem safe to assume that the principles of the mass law apply. Assuming that they do, it would follow that an increase, either of the oxidizing or reducing agent, the other in the meantime remaining constant, would lead to an increase in one or more of the end-products. If it be further assumed that in either case there would be an increase in the concentration of picramic acid, whether or not that of any or all of the other products increased, the observed discordant results would be at least partially accounted for. That is, bloods containing abnormally large amounts of sugar or an abnormally small amount of protein could be presumed to indicate a higher concentration of sugar with this method than would be the case with other methods, in which the balance between the oxidizing and reducing agents is not so easily disturbed.

To study this point experimentally determinations were made on sugar solutions containing (in 4.0 ml.) the glucose which theoretically would be present in the filtrates from 0.64 ml. of bloods containing respectively, 50, 75, 125, 175, and 200 mg. of sugar per 100 ml. of blood. The standard, with which each was compared, contained 0.64 mg. of glucose. The amount of the picrate-picric acid solution, in all cases, was the same as in the standard, 4.0 ml.

From Table I, it is evident that within a range of from 75 to 125 mg. per 100 ml., that is, within 25 per cent of the standard, there is a fairly close correspondence between the glucose actually

present and the glucose indicated by the picramic acid. The maximum error within this range is 1.3 per cent. As the concentration of glucose in the solution was further increased, however, more sugar was indicated than was actually present, there being a positive error of from 3 to 5 per cent. Likewise in the lower part of the range, when 50 mg. of sugar were present, only 47.9 were indicated, a loss of approximately 4 per cent. It seems from the nature of the experiment that the variations in the concentration of glucose, in the unknown from that of the standard, contribute to these discrepancies through the mass effect indicated

TABLE I.

*Comparison of Solutions of Various Concentrations of Glucose with a Standard Solution Containing 0.64 Mg. The Effect of Concentration of Sugar upon the Amount of Picramic Acid Formed in the Lewis-Benedict Method of Determination of Sugar in the Blood.*

No. of determinations.	Glucose to indicate blood sugar concentration in mg. per 100 ml.		Error: Deviation from glucose actually present.	
	Present.	Indicated.	mg.	per cent
	25.0	Colors could not be matched.		
6	50.0	47.9±0.5	-2.1	-4.2
4	75.0	74.0±0.8	-1.0	-1.3
4	100.0	100.1±0.2	+0.1	+0.1
4	125.0	125.2±0.5	+0.2	+0.2
8	150.0	156.1±0.7	+6.1	+4.1
4	175.0	181.5±1.4	+6.5	+3.7
8	200.0	210.0±0.9	+10.0	+5.0

in the above discussion. To study this point still further, solutions containing glucose equivalent to that in the filtrates from 0.64 ml. of bloods containing from 100 to 300 mg. per 100 ml. of blood were compared with a standard containing 1.24 mg. of glucose, *i.e.* double the ordinary strength and corresponding to a blood of 200 mg. per 100 ml.

From Table II it will be seen that, within a range of from 150 to 275 mg. per 100 ml., which is again roughly equivalent to a range of 25 per cent on either side of the standard of comparison, the greatest error is 1.9 per cent, and as a rule the error is much less than this. On the other hand only 90.7 mg. were recovered from

the 100 mg. solution, a loss of 9 per cent; while the 300 mg. solution indicated a concentration of 311.1 mg., a gain of nearly 4 per cent. The behavior of the 100 mg. solution when compared with the different standards is significant. It is of interest to point out at this time that with the advent of insulin and hypoglycemia the possible effect of low concentrations also becomes of importance.

In these two series of experiments, all solutions, containing a much greater concentration of glucose than did the standard of comparison, gave results which indicated a concentration greater than was known to be actually present, while all solutions con-

TABLE II.

*Comparison of Solutions Containing Various Amounts of Glucose with a Standard Solution Containing 1.28 Mg. Which Is Equivalent to a Blood Sugar of 200 Mg. per 100 Ml. The Purpose is to Show the Effect of the Concentration of Glucose upon the Amount of Picramic Acid Formed.*

No. of determinations.	Glucose present.	Glucose found.	Error: Deviation from glucose actually present.	
	Equivalent to blood sugar.			
	<i>mg. per 100 ml.</i>	<i>mg. per 100 ml.</i>	<i>mg.</i>	<i>per cent</i>
4	100.0	90.7 $\pm$ 1.3	-9.3	-9.3
4	150.0	147.2 $\pm$ 0.8	-2.8	-1.9
4	175.0	176.1 $\pm$ 0.2	+1.1	+0.6
4	200.0	199.8 $\pm$ 0.3	-0.2	-0.1
6	225.0	228.1 $\pm$ 1.1	+3.1	+1.4
4	250.0	250.4 $\pm$ 0.4	+0.4	+0.2
4	275.0	277.5 $\pm$ 1.1	+2.5	+0.9
5	300.0	311.1 $\pm$ 0.8	+11.1	+3.7

taining relatively much less glucose than the standard showed a corresponding loss in the amount indicated by the results of the determinations. Every case tested in this manner agreed with the results recorded in these two series. Hence it seems justifiable to conclude that the abnormally high results obtained by this method, as compared with results obtained by other methods, on bloods high in sugar content, may be due, in part at least, to this factor. That is, that the amount of picramic acid formed cannot be assumed to vary directly with the amount of glucose present, except over a very narrow range. This assumption is fundamental to all colorimetric methods of this type, and constitutes a weakness which must not be overlooked

Turning now to the effect of varying the concentration of the picric acid upon the amount of picramic acid formed, we carried out the following experiments. If the protein of the blood were decreased by 50 per cent the picric acid in the blood filtrate would be increased by an amount equivalent to that present in 3.0 ml. of the picrate-picric acid solution, and each 8.0 ml. portion of this filtrate taken for analysis would contain picric acid equivalent to

TABLE III.

*Effect of Varying Amounts of Picric Acid upon the Amount of Picramic Acid Formed with a Fixed Amount of Glucose.*

No. of determinations.	Glucose present 0.64 mg.	Glucose indicated.	Picrate-picric acid solution used.	Deviation from glucose actually present.
	Equivalent to blood sugar.			
	mg. per 100 ml.	mg. per 100 ml.	ml.	mg.
4	100.0	95.1 $\pm$ 0.5	3.0	-4.9
3	100.0	100.0 $\pm$ 0.5	4.0	$\pm$ 0.0
4	100.0	105.6 $\pm$ 1.0	5.0	+5.6
4	100.0	113.0 $\pm$ 1.2	5.5	+13.0

TABLE IV.

*Comparison of the Behavior of Exose and Pure Glucose of Equal Concentration, as Determined by Rotation, Towards Benedict's Blood Sugar Reagent.*

No. of determinations.	Glucose in solution. 0.64 mg. equivalent to 100 mg. per cent.	Glucose indicated.	Type of sugar used.
4	100.0	100.1 $\pm$ 0.2	Exose.
3	100.0	100.0 $\pm$ 0.5	Glucose 2.
4	200.0	209.1 $\pm$ 1.0	Exose.
4	200.0	213.3 $\pm$ 1.9	Glucose 2.

that in 5.0 ml. of the original solution rather than to that in 4.0 ml. as used in the standard tube, an increase of 25 per cent.

To determine the effect of such an increase in the amount of picric acid present, several samples, each containing 0.64 mg. of glucose in 4.0 ml. of water, were prepared. These samples were treated with varying amounts of the picrate-picric acid solution and after reduction were compared with a sample prepared in the standard manner. From Table III, in which the results of the experiment are recorded, it is apparent that any increase in the

amount of picric acid present in the unknown might well be responsible for an indication of extra sugar when compared with a normal standard.

In our hands the method has been reasonably precise; with the optimum concentrations the precision was about  $\pm 1$  per cent. Different observers in the laboratory were able to check each others readings within about the same range. Table IV shows the essentially similar reducing powers of "Exose" and of purified glucose when determined by this method.

TABLE V.

*Behavior of the Folin-Wu Blood Sugar Method towards Various Concentrations of Glucose.*

No. of determinations.	Glucose present.		Glucose indicated: Blood sugar equivalent.	Error: Deviation from glucose actually present.	
	mg.	Blood sugar equivalent. mg. per 100 ml		mg.	per cent
4	0.05	25.0	25.1 $\pm$ 1.2	+0.1	+0.4
4	0.15	75.0	74.9 $\pm$ 0.3	-0.1	-0.1
4	0.20	100.0	99.9 $\pm$ 0.7	-0.1	-0.1
4	0.25	125.0	125.9 $\pm$ 1.2	+0.9	+0.7
4	0.35	175.0	176.4 $\pm$ 2.1	+1.4	+0.8
5	0.45	225.0	228.7 $\pm$ 5.6	+3.7	+1.6
5	0.55	275.0	274.4 $\pm$ 2.8	-0.6	-0.2

### *C. Folin-Wu Method.*

The Folin-Wu (4, 5) method in a sense stands midway between that of Benedict and that of Shaffer and Hartmann, since it is at the same time a colorimetric and a copper reduction method. We found it to give very accurate and precise results over an equivalent range of from 25 to 275 mg. per 100 ml. of blood, when used with solutions of pure glucose, the greatest error, 1.6 per cent, occurring at the upper end of the range. See Table V.

The manipulation we found to be easy, the solutions required are not difficult to prepare, and the colors obtained are of a quality and intensity which render them easy to compare. The method is also extremely sensitive. We were able to detect, and to measure, within 0.4 per cent, as little as 0.05 mg. of glucose, absolute weight. A solution of Exose which was equivalent to 0.100 per cent, as

determined by rotation, and which had a reduction value of  $0.0997 \pm 0.0011$  per cent by the Quisumbing-Thomas method, was used as the unknown and was compared with a standard solution prepared as described by the authors of the method from Sample 2 described above.

### *D. Hagedorn Method.*

The Hagedorn (Höst and Hatlehol (8)) method is an iodometric method in which the oxidizing agent is potassium ferricyanide. The solution also contains potassium iodide. After reduction the solution is acidified and the unreduced excess of ferricyanide reacts with the hydriodic acid, formed on acidification, to liberate

TABLE VI.

*Determination of Pure Glucose in Water Solutions by the Hagedorn Method.*

No. of determinations.	Glucose present.		Glucose indicated: Blood sugar equivalent.	Error: Deviation of indication from glucose actually present.	
	mg.	Blood sugar equivalent. mg. per 100 ml			
5	0.025	25.0	$25.3 \pm 1.6$	+0.3	+1.2
6	0.050	50.0	$49.6 \pm 2.3$	-0.4	-0.8
6	0.075	75.0	$74.4 \pm 3.0$	-0.6	-0.8
9	0.100	100.0	$101.4 \pm 1.8$	+1.4	+1.4
6	0.125	125.0	$124.8 \pm 1.8$	-0.2	-0.2
6	0.175	175.0	$177.0 \pm 2.1$	+2.5	+1.4
11	0.225	225.0	$237.7 \pm 6.7$	+12.7	+5.6
11	0.275	275.0	$292.7 \pm 4.8$	+17.7	+6.4

free iodine. The amount of iodine so liberated is determined by titration with  $N/200$  sodium thiosulfate. The method was found to be accurate over a range of from 25 to 200 mg. per 100 ml. Above the latter concentration, however, we were unable to confirm the authors' table. See Table VI.

The main advantages of this method over the ones more commonly used in this country appear to be that it requires but 0.10 of a ml. of blood and that it avoids the use of copper. This latter point would be of advantage only in special cases as for instance where copper is precipitated, as occasionally occurs with the filtrates of blood from the non-mammals. However, the facts that only 0.01 ml. of the  $N/200$  thiosulfate solution is equivalent



to about 1.8 mg. of glucose per 100 ml. of blood, and that the error in determining the end-point is of the same order of magnitude, make the precision of this method somewhat lower than that of the other methods studied. It possesses, on the other hand, extreme sensitivity, 0.025 mg. being readily indicated. For this reason it would have especial value where only small amounts of sugar are available for determination.

Because of the high dilution of the thiosulfate and the great significance of very small amounts of it, the sensitivity of the starch preparation used as the indicator becomes of considerable importance. It is also of importance that all of the solutions be freshly made, as was indeed recommended by the original author.

#### *E. Shaffer-Hartmann Method.*

This method makes use of the same blood filtrate as is used in that of Folin and Wu. 5 ml. of this filtrate (equivalent to 0.5 ml. of blood) are treated with an equal volume of the Shaffer-Hartmann reagent and placed in a boiling water bath for 15 minutes. At the end of this time the tubes are removed from the bath and cooled in running water. Each is then acidified and after a short time, allowed for the completion of the reaction, is titrated with  $N/200$  thiosulfate solution. The value obtained, subtracted from that of a blank titration, gives the thiosulfate equivalent to the iodine which was used in reoxidizing the cuprous ion, and hence is a measure of the sugar which was originally present.

To study the behavior of this method, solutions of pure glucose were so made that 5 ml. of the respective solutions contained sugar corresponding to that of the filtrates from bloods in which the sugar ranged from 10 to 325 mg. per 100 ml. The results, expressed in ml. of  $N/200$  thiosulfate, are given in Tables VII, VIII, and IX. In these tables the number of determinations which were made, the absolute quantities of glucose present, the concentrations of glucose in the blood to which these quantities correspond, as well as the thiosulfate equivalent as given in the tables of Shaffer and Hartmann are given. The tables show the method to be consistent with itself from day to day and with different solutions and different samples of glucose.

All of our determinations were made under conditions as nearly simulating those described by Shaffer and Hartmann as was pos-

sible, probably much more closely than would be the case in ordinary routine work. The titrations were made in a darkened room with the light from a Chalet colorimeter lamp reflected through the solution from below. The light for titration was thus con-

TABLE VII.  
*Determination of Pure Glucose in Water Solution by the Shaffer-Hartmann Method.*

Glucose present.		N/200 thiosulfate equivalent to glucose present.						
	Blood sugar equivalent.	Shaffer-Hartmann table.	Actual titration.					
			Samples of glucose used.					
			No. 2.	No. 1.	No. 1.	No. 2.	No. 2.	No. 2.
mg.	mg. per 100 ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
0.05	10.0	0.25	0.05	0.03		0.06		
0.10	20.0	0.50	0.07	0.07		0.11		
0.15	30.0	0.75	0.42	0.37		0.42		
0.20	40.0	1.00		0.70	0.76	0.74		
0.25	50.0	1.31	1.16	1.07	1.12	1.11		
0.50	100.0	3.15	3.08	2.97	3.01	3.03	3.04	3.03

TABLE VIII.  
*Determination of Pure Glucose in Water Solution by the Shaffer-Hartmann Method.*

Glucose present.		N/200 thiosulfate equivalent to glucose present.				
	Blood sugar equivalent.	Shaffer-Hartmann table.	Actual titration.			
			Samples of glucose used.			
			No. 2.	No. 1.	No. 2.	No. 2.
mg.	mg. per 100 ml.	ml.	ml.	ml.	ml.	ml.
0.375	75.0	2.25	2.07	2.00	2.02	
0.625	125.0	4.12	4.00	3.94	3.93	
0.875	175.0	5.96	5.91	5.92	5.86	
1.125	225.0	7.92	7.89	7.82	7.86	
1.375	275.0	9.79	9.81	9.81	9.86	9.89
1.625	325.0	11.83	11.93	11.94	11.92	

sistent from day to day. We feel that the end-point may be determined a little more precisely by reflecting the light through the solution in this manner than by the ordinary procedure. It should be emphasized, however, that the discrepancies between

our results and the table of Shaffer and Hartmann, which were revealed, in no way depended upon this method of lighting but have time and again shown themselves when the titration was done in ordinary daylight.

While the differences between our values and those given by the authors of the method are not large over the range of normal blood sugar, possibly not even significant, it will be noted that there is actual agreement only in the extreme upper part of the range. In the region of interest during insulin convulsions the difference

TABLE IX.

*Compilation of All of Determinations of Pure Glucose in Water Solution by the Shaffer-Hartmann Method.*

No. of determinations.	Glucose present.		N/200 thiosulfate required.		Mean deviation.	Deviation from Shaffer-Hartmann table.
		Blood sugar equivalent.	Shaffer-Hartmann table.	By titration.		
	mg.	mg. per 100 ml.	ml.	ml.	ml.	per cent
14	0.050	10.0	0.25	0.05	±0.03	80
14	0.100	20.0	0.50	0.08	±0.04	84
14	0.150	30.0	0.75	0.40	±0.04	47
11	0.200	40.0	1.00	0.73	±0.05	27
18	0.250	50.0	1.31	1.12	±0.05	14
12	0.375	75.0	2.25	2.03	±0.04	9.8
22	0.500	100.0	3.15	3.03	±0.04	3.6
11	0.625	125.0	4.12	3.95	±0.04	4.1
12	0.875	175.0	5.96	5.90	±0.04	1.0
12	1.125	225.0	7.92	7.86	±0.05	0.8
15	1.375	275.0	9.79	9.85	±0.06	0.6
12	1.625	325.0	11.83	11.93	±0.07	0.8

becomes significant and in the lower portions of the range the agreement between our results and those of Shaffer and Hartmann breaks down entirely.

Our attention was first directed to a possible inaccuracy in this range by Miss Hiller<sup>1</sup> of the Hospital of the Rockefeller Institute who had made simultaneous determinations with the Shaffer-Hartmann and the Hagedorn methods upon glycolyzing blood. The point made by Miss Hiller was that during glycolysis or fer-

<sup>1</sup> This work has since been published (7).

TABLE X.

*Amounts of Glucose and Glucose Concentration of Blood Corresponding to Various Amounts of N/200 Thiosulfate. Shaffer-Hartmann Method.*

Thiosulfate used. N/200	Actual glucose indicated.	Glucose in 100 ml. of blood.	Thiosulfate used. N/200	Actual glucose indicated.	Glucose in 100 ml. of blood.	Thiosulfate used. N/200	Actual glucose indicated.	Glucose in 100 ml. of blood.	Thiosulfate used N/200	Actual glucose indicated.	Glucose in 100 ml. of blood.
ml.	mg.	mg.	ml.	mg.	mg.	ml.	mg.	mg.	ml.	mg.	mg.
0.10	0.100	20	3.10	0.509	102	6.10	0.902	180	9.10	1.282	256
0.20	0.120	24	3.20	0.523	105	6.20	0.914	183	9.20	1.294	259
0.30	0.135	27	3.30	0.537	107	6.30	0.926	185	9.30	1.306	261
0.40	0.150	30	3.40	0.551	110	6.40	0.938	188	9.40	1.318	263
0.50	0.165	33	3.50	0.565	113	6.50	0.950	190	9.50	1.330	266
0.60	0.180	36	3.60	0.578	116	6.60	0.963	193	9.60	1.343	269
0.70	0.195	39	3.70	0.591	118	6.70	0.976	195	9.70	1.356	271
0.80	0.210	42	3.80	0.604	121	6.80	0.989	197	9.80	1.369	273
0.90	0.220	44	3.90	0.617	123	6.90	1.002	200	9.90	1.382	276
1.00	0.235	47	4.00	0.630	126	7.00	1.015	203	10.00	1.395	279
1.10	0.245	49	4.10	0.643	129	7.10	1.028	205	10.10	1.407	281
1.20	0.260	52	4.20	0.656	131	7.20	1.041	208	10.20	1.419	283
1.30	0.275	55	4.30	0.669	134	7.30	1.054	211	10.30	1.431	286
1.40	0.290	58	4.40	0.682	136	7.40	1.067	213	10.40	1.443	289
1.50	0.300	60	4.50	0.695	139	7.50	1.080	216	10.50	1.455	291
1.60	0.315	63	4.60	0.708	141	7.60	1.093	219	10.60	1.467	293
1.70	0.330	66	4.70	0.721	144	7.70	1.106	221	10.70	1.479	296
1.80	0.345	69	4.80	0.734	147	7.80	1.119	223	10.80	1.491	298
1.90	0.355	71	4.90	0.747	149	7.90	1.132	226	10.90	1.503	301
2.00	0.370	74	5.00	0.760	152	8.00	1.145	229	11.00	1.515	303
2.10	0.383	77	5.10	0.773	155	8.10	1.157	231	11.10	1.527	305
2.20	0.396	79	5.20	0.786	157	8.20	1.170	234	11.20	1.539	307
2.30	0.409	82	5.30	0.799	159	8.30	1.182	236	11.30	1.551	310
2.40	0.422	84	5.40	0.812	162	8.40	1.193	239	11.40	1.563	313
2.50	0.435	87	5.50	0.825	165	8.50	1.205	241	11.50	1.575	315
2.60	0.447	89	5.60	0.838	167	8.60	1.218	243	11.60	1.587	317
2.70	0.459	92	5.70	0.851	170	8.70	1.231	246	11.70	1.599	319
2.80	0.471	94	5.80	0.864	173	8.80	1.244	249	11.80	1.611	322
2.90	0.483	97	5.90	0.877	175	8.90	1.257	251	11.90	1.623	325
3.00	0.495	99	6.00	0.890	178	9.00	1.270	254	12.00	1.635	327

mentation experiments the Hagedorn method still indicated a concentration of about 20 mg. per 100 ml. when the Shaffer-Hartmann method first registered zero. This was soon and abundantly confirmed in our own laboratory. It was in fact this circumstance which led to the present study. In addition to studies in glycolysis the lower portions of the table are of importance in glycogen determinations, in the study of blood sugar in the invertebrates, and doubtless in other phases of carbohydrate metabolism.

Table X is constructed from the results recorded in Tables VII and VIII by interpolation and is offered as a substitute for Table II, p. 380, of Shaffer and Hartmann. It is very strongly urged, however, that each investigator who uses this method construct his own table, rather than use either ours or the one of the original authors.

## *II. Results of the Work with Blood.*

A few experiments have been carried out with blood in order to determine to what extent the results described in Part I of this paper may explain the variations which have been noted in practical blood sugar analyses.

Oxalated beef blood was used in these experiments. As soon as the blood was received from the slaughter-house it was strained through coarse gauze to remove any small clots which might have formed and then it was kept for use at 2°C., except while samples were being removed for analysis. The sugar content was first determined simultaneously by each of the four methods. The results are given in Table XI. From this table it will be seen that the first three methods gave almost identical results but that the Shaffer-Hartmann method, using their own table, indicated but 117 mg. of glucose per 100 ml. of blood as against 120 or more as determined by the other methods. If our table is used the value is brought up to 120 and compares favorably with that indicated by the other methods.

In order to determine the behavior of the Shaffer-Hartmann method in the lower ranges, simultaneous determinations were made on 5 ml. of the blood filtrate and on 2 ml. which had been diluted to 5 by the addition of 3 ml. of water. From 2 ml. 40 per cent of the glucose indicated in the 5 ml. samples should be

recovered. Two trials were made, with the results shown in Table XII. It is evident from these experiments, quite apart from our other work and quite in accord with the work of Hiller, Linder, and Van Slyke, that the table of Shaffer and Hartmann

TABLE XI.

*Sugar Recovered from a Single Sample of Blood by the Four Different Methods.*

Method.	No. of determinations.	Sugar content of blood.
		<i>mg. per 100 ml.</i>
Benedict.....	4	121.4 $\pm$ 1.2
Folin-Wu.....	4	122.9 $\pm$ 1.6
Hagedorn.....	5	120.2 $\pm$ 2.3
Shaffer-Hartmann. (Authors' table.).....	4	117.1 $\pm$ 1.2
“ Our “ .....	4	120.1 $\pm$ 1.2

TABLE XII.

*Comparison of the Behavior of the Shaffer-Hartmann Method when Used with Two Different Amounts of Glucose in a Blood Filtrate.*

	No. of deter- minations.	N/200 thio- sulfate.	Mg. glucose per 100 ml. of blood, calculated from:	
			Shaffer-Hartmann table.	Table X.
		ml.		
Sample A.				
5 ml. of blood filtrate....	4	3.54	110.1 $\pm$ 1.3	113.9 $\pm$ 1.3
40 per cent of above.....			44.0 $\pm$ 1.3	45.6 $\pm$ 1.3
2 ml. of blood filtrate....	3	0.86	34.5 $\pm$ 1.2	43.5 $\pm$ 1.2
Difference.....			9.5 = -21%	2.1 = -4.6%
Sample B.				
5 ml. of blood filtrate....	4	3.27	103.0 $\pm$ 1.2	106.5 $\pm$ 1.2
40 per cent of above.....			41.2 $\pm$ 1.2	42.6 $\pm$ 1.2
2 ml. of blood filtrate....	4	0.88	35.0 $\pm$ 2.1	43.7 $\pm$ 2.1
Difference.....			6.2 = -15%	1.1 = +2.6%

is not consistent over the range here used. It is apparent, also, that at the blood sugar level of insulin convulsions, which was approximated in the 2 ml. samples, the error arising from the use of the original tables is considerable, of the order of 15 or 20 per cent of the total sugar present.

In Part I it was shown that the amount of glucose which is indicated by Benedict's method depends upon the relative amounts of glucose in the standard and in the unknown solution and upon the amount of picric acid present (Table III). It might be supposed that any large decrease in the amount of protein present would increase the amount of picric acid left to oxidize the glucose.

To test this the sugar in a fresh sample of beef blood was determined by the Benedict method (as described by the author) and found to be 107 mg. per 100 ml. of blood. A solution of pure glucose of this concentration was prepared in 0.9 per cent sodium chloride solution. 70 ml. of the original blood were diluted to 100 ml. with this solution. In this manner the sugar concentration was left unchanged while that of the protein was reduced 30 per cent. The amount of picric acid in the filtrate should then be increased by 30 per cent of the amount usually removed in precipitating the protein; that is, 30 per cent of the picric acid which would be in 7.0 ml. of the picrate-picric acid solution. This would increase the picric acid in an 8.0 ml. portion of the filtrate by an amount equal to that in 0.63 ml. of the picrate-picric acid solution, an increase of 16 per cent in the available picric acid. We have shown, Table III, that an increase of 25 per cent in the available picric acid causes the indication of 5.6 per cent of extra glucose, so that the increase here would be 3.6 per cent of 107 or 3.8 mg. of extra glucose per 100 ml. of blood.

$$\text{Calculation: } \frac{16}{25} \times 0.056 \times 100 = 3.6.$$

We would expect, then, to find indications for about 110 mg. of sugar per 100 ml. of the blood which had been diluted. The following shows the results of such an experiment.

Original blood unmodified, Benedict method.....	106.7 ± 2.8
Protein decreased 30 per cent. Theoretical increase in sugar indicated.....	3.8

Theoretical indication for diluted blood .....	110.5
Sugar content of diluted blood as actually found.....	111.0 ± 1.6

From the figures just given it is evident that our supposition is at least fair, and that any considerable decrease in the blood proteins may be expected to increase the apparent sugar content of the blood when the latter is determined by this method.

To 100 ml. of this same original blood 100 mg. of pure glucose were added, making a total glucose content of the blood of 0.2067 per cent. From Table IV we would expect 14 mg. of extra glucose to be indicated, so that by this method there should be an indicated concentration of  $206.7 + 14.0 = 220.7$  mg. per 100 ml. of the modified blood. The experimental figures are tabulated below.

Original blood, unmodified Benedict method.....	106.7 $\pm$ 2.8
Modified blood, 100 mg. glucose added to the above.....	206.7
Increase expected from results in Part I.....	14.0
Expected indication.....	220.7
Actual indication.....	221.6 $\pm$ 1.4

It is thus evident that the results with the blood agree favorably with those obtained from the solutions of pure glucose in Part I of the present paper. In further support of this conception are certain results obtained by Hst and Hatlehol (8) who compared the Bang, Hagedorn, Folin-Wu, and the Myers and Baily modification of the Lewis-Benedict methods. They determined the glucose of normal human bloods and found that the four methods agreed fairly closely. When glucose was added, however, so that the glucose content was actually in the neighborhood of 0.200 per cent, the Benedict method usually indicated an excess of from 6 to 14 mg. over the other methods, which agrees well with our results.

#### DISCUSSION.

We have not attempted to define the nature of the reducing substances in the blood but have considered the behavior of the several methods, first towards a solution of pure glucose and then towards the effective reducing substances of the blood considered as pure glucose.

It has been shown that errors may arise during the progress of blood sugar determinations which are inherent in the method used. These errors may be due to the relative amounts of the reacting substances present in the system, or to other causes. It has been shown that some of the discrepancies between different blood sugar methods need not necessarily be ascribed, as is so often done, to hypothetical reducing substances other than glucose, which are presumed to be in the blood. Thus the extremely high sugar content sometimes indicated by Benedict's method for diabetic



blood need not necessarily be ascribed to creatinine but may simply be the direct result of the high glucose concentration.

An increase in the oxidizing agent may likewise increase the amount of sugar indicated. It is possible that both of these effects and especially the latter might be reduced if the final concentration of the oxidizing agent could be increased. In line with this suggestion it is interesting to note that Calvert (2, 3) found the Folin-Wu method to behave in a somewhat similar manner when the alkaline-copper-tartrate solution was diluted, that is, when the concentration of the oxidizing agent was reduced. Stanford and Wheatley (12) point out that a large excess of the copper tartrate is necessary if the reduction is to be strictly parallel to the amount of sugar present. Apparently one of the factors in the success of the Folin-Wu method lies in the great solubility of the oxidizing agent chosen.

Shaffer and Hartmann's method has been found to be very satisfactory to work with when its limitations are known and recognized. The technique is simple and the results are consistent. A lack of sensitivity to very low concentrations of glucose however, distinctly limits its field of usefulness. The origin of this loss of sensitivity apparently arises from the introduction of the iodine salts or of the oxalate, as otherwise their solution is very similar to that of Folin and Wu, which is apparently much more sensitive.

Haskins and Holbrook (6) have recently offered a modification of this method for clinical use. Altogether their modifications seem to have decreased the reliability of the method without contributing much towards its convenience.

#### SUMMARY.

1. The behaviors of four different methods for the determination of blood sugar have been compared.

2. The Folin-Wu and Hagedorn methods were found to be accurate and fairly precise when used to determine the concentrations of pure glucose solutions approximating those encountered in blood analysis.

3. It was found that an increase in the concentration of either the sugar or of the picric acid in the Benedict method would lead to high results, and this is offered as at least a partial explanation

of the high results obtained by this method in certain pathological bloods.

4. The Shaffer-Hartmann method was found to be reliable and satisfactory for concentrations above about 25 mg. per 100 ml. of blood. Certain corrections also appear to be necessary to their table. A substitute table is submitted.

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# **TITRIMETRIC DOUBLE HYDROGEN OR QUINHYDRONE ELECTRODE SYSTEMS FOR HYDRION DETERMINA- TION; APPLICATIONS TO URINE AND BLOOD.**

BY GEORGE H. MEEKER AND BERNARD L. OSER.

*(From the Laboratories of Biochemistry of the Philadelphia General Hospital  
and the Graduate School of Medicine of the University  
of Pennsylvania, Philadelphia.)*

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In this paper are presented the details of a simplified method for determining hydron concentration electrometrically, requiring only the use of electrodes and a galvanometer, but dispensing with the potentiometer and the standard cell.

Klopsteg (1, 2), in 1920, suggested the use of the double hydrogen electrode in electrometric titrations. The apparatus he used consisted of two hydrogen electrodes, one immersed in a buffer solution of known pH, the other in the solution to be titrated to that pH. A galvanometer with protective resistance and a tapping key completed the circuit. Klopsteg specifically stated, however, that the method "can be used only for titrating and that the titration can be carried only to the end-point which is determined by the standard solution" and that for measurement of pH a potentiometer must be used. Our purpose is to demonstrate the applicability of the same arrangement for the measurement of pH.

In the course of the experimental work on the double hydrogen electrode system it was realized that the step in the manipulative process that required special care was the preparation of the electrodes themselves, and that this factor could be eliminated by the use of quinhydrone electrodes. This permits the use of un-platinized metallic electrodes, and dispenses with hydrogen generation, bubbling, etc. The quinhydrone system thus has the further advantage of being adaptable to the measurement of the

pH of CO<sub>2</sub>-containing fluids such as are met with in biological work.

Briefly, the method consists in balancing a potential set up in a half-cell containing a solution of unknown pH, with that produced by a known mixture of two solutions of a buffer pair in the other half-cell, the proportions of the latter constituting a measure of the pH of the unknown solution, inasmuch as at zero deflection of the galvanometer the hydrogen ion concentrations of the solutions in the opposing half-cells are the same.

*Apparatus.*—For double hydrogen electrode work any convenient type of bubbling electrode, such as described in Clark's book (3), could be used. In the present experiments the Hildebrand electrode was modified to increase its stability, thus eliminating electrode supports. The jacket was shorter and had a conical bell. The electrodes themselves were platinized in the customary manner (Clark), rotating slowly to insure uniformity of the deposit.

The electrodes used in the quinhydrone system consisted simply of heavy platinum or 24 carat gold wire sealed through glass tubes about 10 cm. long and 2 mm. internal diameter. The gold wires were sufficiently long to permit direct contact with the copper leads, while in the platinum electrodes contact was made through mercury.

Saturated potassium chloride was used in the salt bridge. Hydrogen was delivered from a cylinder and passed through alkaline pyrogallol and distilled water.

An inexpensive galvanometer (Leeds and Northrup potentiometer galvanometer No. 2320d) was found to be sufficiently sensitive for this work. In fact the sensitivity of the galvanometer recommended for the type K potentiometer equipment exceeds that of the titration process and is therefore less desirable. Additional resistance in the circuit is not really necessary from the point of view of protection to the galvanometer. If the stops in the instrument are so adjusted as to confine the deflection of the pointer within scale limits such resistance may be dispensed with entirely. A tapping key was also introduced into the circuit.

*Procedure for the Double Hydrogen Electrode System.*—The solution whose pH was to be measured was introduced into one of the half-cells (reference cell) and a measured volume of one of the

buffer pair<sup>1</sup> into the other. The hydrogen electrodes were immersed in the solutions and connected with the hydrogen supply. Hydrogen was allowed to bubble through the solutions sufficiently to saturate them as well as the electrodes. The time necessary for this was previously determined as complete saturation is of fundamental importance. 15 minutes were allowed for this step, and both solutions were kept saturated with hydrogen throughout the procedure. The salt bridge was then introduced and the other solution of the buffer pair delivered into the titration cell from a burette. The key was tapped at intervals, more frequently as the extent of the deflection of the galvanometer diminished. At zero deflection a reading of the burette was made. The electrode from the reference cell was then immersed in the buffer solution and the equality of the electrodes confirmed by producing zero deflection of the galvanometer. Following this the electrode was returned to its original solution and another drop or two of buffer added to produce deflection in the opposite direction.

The necessity for equivalence of the two hydrogen electrodes cannot be emphasized too strongly. They should be platinized in exactly the same manner, completely saturated with hydrogen, and checked for parity by zero deflection when immersed together in one cell, before the titration is begun.

The volumes of the buffer pair, the pH of whose mixture equals that of the unknown solution, were now known. Curves were constructed from the data for each buffer system (the most important of which are found in Clark (3)) in which the proportion of one of the solutions in the final mixture was plotted against the pH. From these curves was read directly the pH of the unknown solution. Examples of such curves are given in Fig. 1.

In Table I are given the results obtained by using as "unknown" solutions, standard buffer solutions of definite pH, and balancing against them the buffer solutions as indicated above. The final values were read from the curves in Fig. 1.

<sup>1</sup> The selection of the buffer solution depends, of course, on the range of pH in which the unknown solution falls. This can be readily estimated by the use of a set of indicators such as those of Clark and Lubs. For instance, if a solution is "alkaline" to methyl red and "acid" to phenol red, its pH must fall somewhere between the ranges of these two indicators, namely pH 6 to pH 7. In this case buffer solutions are selected covering this range. If the indicators of Clark and Lubs are used, the color chart in Clark's book will be found helpful.

*Procedure for the Quinhydrone Electrode System.*—The first practical application of the quinhydrone electrode to the determination of pH was due to Biilmann (4). The theoretical considerations upon which he based its use were founded on the conception of the electrode being in effect a hydrogen electrode at low partial pressure of hydrogen. Recently the electrochemical view-point has received emphasis (3, 5) in which the oxidation-

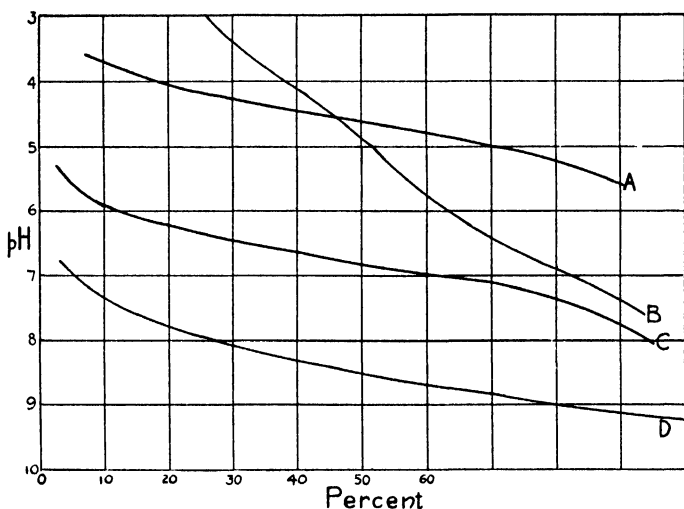


FIG. 1.

- A, Walpole's acetate mixtures (per cent sodium acetate).  
 B, McIlvaine's phosphate-citric acid mixtures (per cent disodium phosphate).  
 C, Sørensen's phosphate mixtures (per cent disodium phosphate).  
 D, Palitsch's borax-boric acid mixtures (per cent borax).

reduction potential is attributed to an effective electron pressure, resulting from the equilibrium



In the determinations here reported it should be emphasized that potentials were not measured but were simply balanced.

Quinhydrone was prepared from hydroquinone by the action of ferric alum, following the method of Biilmann and Lund (6).

The trace of iron salt remaining was found not to interfere with the determinations.

The unknown solution and the measured volume of one solution of the buffer pair were introduced into their respective beakers, and just preliminary to establishing the connection with the salt

TABLE I.  
*Double Hydrogen Electrode System.*

"Unknown" cell (standard buffer).	Titration cell.				Ratio.*	pH found.	Difference.
	Buffer A.		Buffer B.				
pH		cc.		cc.	per cent		
4.03	0.1 N HOAc	8.0	0.1 N NaOAc	2.03	20.2	4.02	-0.01
4.25	0.1 " "	10.0	0.1 " "	4.05	28.8	4.26	+0.01
4.60	0.1 " NaOAc	10.0	0.1 " HOAc	10.40	49.0	4.60	0.00
4.98	0.1 " HOAc	3.0	0.1 " NaOAc	7.02	70.0	4.96	-0.02
5.90	M/15 KH <sub>2</sub> PO <sub>4</sub>	20.0	M/15 Na <sub>2</sub> HPO <sub>4</sub>	2.36	10.6	5.92	+0.02
6.00	M/15 "	10.0	M/15 "	1.39	12.2	6.01	+0.01
6.50	M/15 "	10.0	M/15 "	4.80	32.4	6.51	+0.01
7.00	M/15 "	10.0	M/15 "	16.10	61.7	7.01	+0.01
7.10	M/5 KH <sub>2</sub> PO <sub>4</sub>	5.0	M/5 NaOH	3.30	39.8	7.10	0.00
7.20	Universal buffer.†	10.0	M/10 "	10.00		7.20	0.00
7.30	M/15 KH <sub>2</sub> PO <sub>4</sub>	7.0	M/15 Na <sub>2</sub> HPO <sub>4</sub>	23.60	77.1	7.31	+0.01
7.50	Universal buffer.†	10.0	M/5 NaOH	5.75		7.52	+0.02
7.65	M/15 KH <sub>2</sub> PO <sub>4</sub>	5.0	M/15 Na <sub>2</sub> HPO <sub>4</sub>	35.50	87.7	7.64	-0.01
8.10	H <sub>3</sub> BO <sub>3</sub> -NaCl	10.0	M/20 Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub>	4.25	29.8	8.08	-0.02
8.15	"	10.0	M/20 "	5.00	33.3	8.16	+0.01
9.00	M/20 Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub>	10.0	H <sub>3</sub> BO <sub>3</sub> -NaCl	2.60	79.4	8.98	-0.02

\* Ratio, in per cent, of the more basic solution of the buffer pair in the final mixture (Buffer A plus Buffer B).

† The universal buffer solution of Acree and associates, which covers the range pH 1 to 12.

bridge an excess (enough to cover the tip of a spatula) of quinhydrone was added to each solution and stirred thoroughly for about a minute. Since quinhydrone is rather insoluble equilibrium is reached quite readily. The second solution of the buffer pair was then added from a burette as in the foregoing method, the mixture being stirred constantly. The burette reading at zero



deflection was noted and an additional drop or two of solution introduced, whereupon deflection occurred in the opposite direction.

It was noted that, other conditions being the same, greater deflections were produced with platinum electrodes than with gold. Since degree of deflection is the criterion of the method, platinum should therefore be preferred, provided it is inert to the particular system being measured. In some cases, such as in whole blood, the platinum behaves as an oxidative catalyst, thus rendering necessary the use of gold, which is inert in this respect (7).

TABLE II.  
*Double Quinhydrone Electrode System.*

"Unknown" cell (standard buffer).	Titration cell.				Ratio.	pH found.	Difference.
	Buffer A.		Buffer B.				
pH		cc.		cc.	percent		
7.00	M/15 $\text{Na}_2\text{HPO}_4$	10	M/15 $\text{KH}_2\text{PO}_4$	6.8	59.6	6.98	-0.02
7.05	M/15 "	15	M/15 "	7.75	66.0	7.07	+0.02
7.40	M/15 "	10	M/15 "	3.9	71.9	7.40	0.00
7.72	M/15 "	26.5	M/15 "	3.0	89.8	7.71	-0.01
7.75	M/15 "	10	M/15 "	1.00	91.9	7.78	+0.03
7.90	M/15 "	25.0	M/15 "	1.8	93.3	7.92	+0.02
8.04	M/15 "	18.9	M/15 "	1.0	95.0	8.02	-0.02
8.05	M/15 "	25.0	M/15 "	1.2	95.4	8.07	+0.02
8.16	$\text{H}_3\text{BO}_3\text{-NaCl}$	10.0	M/20 $\text{Na}_2\text{B}_4\text{O}_7$	5.15	34.0	8.19	+0.03
8.50	"	10.0	M/20 "	10.55	51.8	8.53	+0.03

The final calculation and readings of pH were made by reference to the same curves as described above. Examples of pH titrations using the quinhydrone system are given in Table II.

The chief limitation to the application of the quinhydrone electrode system rests on its instability in alkaline solution. La Mer and Parsons (5) investigated the relative effects of autoxidation, ionization, and the acid behavior of hydroquinone in alkaline solution by comparing measurements with the hydrogen and quinhydrone electrodes in potentiometric titrations. These authors concluded that pH 8.0 represented the maximum alkalinity

for which the quinhydrone electrode system would be applicable. Schaefer and Schmidt (8) have more recently made a comparative study of the two methods and suggest pH 9.0 as the limit of alkalinity.

Inasmuch as it was our ultimate object to apply the quinhydrone method to blood, preliminary titrations were made with buffer solutions covering the range of pH 7.0 to pH 8.0. These results are tabulated in Table II, and indicate that measurements may be made within this range with reliance. It was observed however that if too much time elapsed during the titration, erroneous results were produced. From 2 to 3 minutes was generally the duration of a complete determination from the time of addition of quinhydrone until the end-point was reached.

The altered activities of quinone and hydroquinone in solutions of high salt concentration preclude the usefulness of the quinhydrone system in such media. Sørensen, Sørensen, and Linderström-Lang (9) have investigated this phase of the problem and have found that it is not appreciable in concentrations lower than  $m/5$ . This factor must be taken into account in the practical application of the procedure to physiologic fluids.

#### DISCUSSION.

The agreement, it will be seen, is quite close in both electrode systems. The sensitiveness of the method is, of course, dependent not only on the size of the drop delivered from the burette, but also on the slope of the titration curve at the particular range involved. The latter may however be increased where greater sensitiveness is desired by extending the coordinates along the pH axis, but this obviously should not be carried beyond the limit of sensitivity of the other factors.

The buffer solutions tabulated in the text-books of Clark (3, or Michaelis (10) have been standardized potentiometrically, and are entirely dependable, provided, of course, the purity of the salts conforms to the standards set by the various investigators. If it is desirable to use as few solutions as possible to cover a wide range of pH, buffer pairs like those of McIlvaine (11) (see Fig. 1) or Acree and associates (12) may be used.

*Applications of the Quinhydrone Electrode to the Determination of pH.*

*Urine.*—Schaefer and Schmidt (8) have reported a long series of experiments on the pH of urine determined potentiometrically, using on the one hand the hydrogen electrode, and on the other the quinhydrone electrode. In the latter case the reference electrode also consisted of a quinhydrone electrode, the solution being 0.1 N HCl-KCl, in the proportion of 1 of the acid to 9 of the salt. The data of these authors also include colorimetric estimations, and all these methods gave concordant results.

In the experiments here reported, colorimetric determinations of the pH of urine were made at dilutions of 1:20, using Clark and Lubs indicators. Walpole acetate mixtures and Sørensen phos-

TABLE III.  
*Comparative pH Determinations of Urine.*

Colorimetric method.	Quinhydrone method.
6.8-7.0	6.98
6.5	6.53
5.2	5.22
6.6	6.64
5.4	5.47
6.8-7.0	6.86
6.6-6.8	6.72

phate mixtures constituted the standard buffer solutions, and these varied by intervals of 0.2 pH.

In order to eliminate the subjective error, where the pH of a specimen lay between two standards, the pH values of the latter were recorded instead of an estimated value.

The electrometric titrations were conducted on urine diluted approximately 1:20, following the technique outlined above. Typical results are presented in Table III.

This method is useful in measuring hydron concentration in cases where the solutions are too deeply colored or turbid, as well as in those cases (*e.g.*, certain urines) where the dichromatic effects of the indicator render colorimetric comparison difficult.

*Blood.*—Success had been claimed for the quinhydrone electrode in the potentiometric estimation of the pH of blood by Corran

and Lewis (13), a calomel cell serving as the reference electrode. Since saturation with quinhydrone without loss of  $\text{CO}_2$  was a necessary step, their use of undiluted whole blood appears to be an objectionable feature. Similar criticism might be applied to the syringe electrode described by Mislowitz (14) for pH measurements of blood.

The experiments of Corran and Lewis were not confirmed by Cullen and Biilmann (15). These authors describe a capillary quinhydrone electrode for estimating the pH of undiluted plasma or serum by means of the potentiometer. Their experience with whole blood however was unsatisfactory.

The titrimetric procedure adopted for the determination of the pH of blood is as follows: The reference cell for the blood was prepared with 10 cc. of physiological saline solution to which was added an excess of quinhydrone. After vigorous stirring, a layer of neutral mineral oil was added and the beaker immersed in a bath at  $38^\circ\text{C}$ . A thermometer was suspended in this reference cell and a gold electrode, which had already been connected to the rest of the circuit, was immersed. When the temperature of the contents of this cell reached  $38^\circ$ , there were added to the titration cell 10 cc. of  $\text{M}/15$  disodium hydrogen phosphate and an excess of quinhydrone, and the other gold electrode was then immersed in it. 0.8 cc. of oxalated whole blood, collected without loss of  $\text{CO}_2$ , was then introduced into the saline under oil and stirred thoroughly for 20 to 30 seconds. The salt bridge connection was then made and the titration with  $\text{M}/15$  potassium dihydrogen phosphate conducted without delay, using a micro burette. During this short space of time the temperature of the diluted blood was maintained at  $38^\circ$  without difficulty. The final pH values were obtained as previously indicated from the Sørensen phosphate curves.

While no altogether satisfactory method for the colorimetric determination of the hydrogen ion concentration of blood exists, that of Cullen (16) makes the closest approach to it. The technical simplification introduced by Hawkins (17) permits the use of small quantities of blood. The only uncertain factor in the method is the empirical correction that must be applied to bring the pH as determined colorimetrically at room temperature to the value obtained electrometrically at  $38^\circ\text{C}$ . according to the equation

$$\text{pH}_{38} = \text{pH}_t + 0.01 (t - 20) - 0.23$$

According to Hastings and Sendroy (18) no correction is necessary if the reading is made with both the standard and the diluted plasma at 38°C. This has been confirmed by Drucker and Cullen (19) but in agreement with these authors we find it more convenient to work at room temperature and apply the correction, which for human blood appears to be quite constant ( $-0.23 \pm 0.04$ ). The original Cullen standards were used, inasmuch as no greater permanence was observed with the bicolor standards of Hastings and Sendroy.

In Table IV are recorded the results by the two methods of some determinations conducted on the blood of hospital patients. Considering the slight individual variations in the Cullen correction the agreement between the two methods is satisfactory.

TABLE IV.  
*Comparative pH Determinations of Blood.*

Colorimetric method.	Quinhydrone method.	Difference.
7.37	7.41	+0.04
7.42	7.39	-0.03
7.41	7.45	+0.04
7.63	7.62	-0.01
7.42	7.38	-0.04
7.36	7.34	-0.02

#### SUMMARY.

1. A simplified electrometric method for the measurement of hydrogen ion concentration is presented based on the use of the double hydrogen electrode or the double quinhydrone electrode, in which the potential of the measured solution in one half-cell is balanced by titrating standard buffer solutions into the other half-cell. The proportions of the latter solutions required to produce equilibrium determine the pH of the unknown solution.

2. Applications of the quinhydrone electrode to the estimation of the pH of urine and blood are described, which give results concordant with the colorimetric methods now in general use.

The authors wish to express their indebtedness to Dr. D. Wright Wilson and to Dr. Glenn E. Cullen for their helpful criticism in the preparation of this paper.

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## THE CORRECTION OF FOLIN-WU BLOOD SUGAR VALUES.

BY BERNARD L. OSER AND WALTER G. KARR.

*(From the Laboratories of Biochemistry of the Philadelphia General Hospital and the Graduate School of Medicine of the University of Pennsylvania, Philadelphia.)*

(Received for publication, November 13, 1925.)

It has been previously recognized that an important source of error in the determination of blood sugar by the method of Folin and Wu (1) rests on the fact that the blue color obtained fails to obey Beer's law, which states that the color intensity varies directly as the concentration. It was for this reason that Folin and Wu included two standard solutions in their method, although accurate results can only be obtained when the readings are close to the settings of either of the standards. The error therefore becomes appreciable not only in the case of high or low blood sugars, but also in the region of 130 to 180 mg. per 100 cc., where it is frequently difficult to interpret the finding.

A partial solution to this difficulty is achieved by using a third standard glucose solution whose concentration lies between the original standards (*i.e.* 2 cc. = 0.3 mg.), but this is not wholly satisfactory for the reason indicated above. Rothberg and Evans (2) advise the use of graduated sugar tubes which permit of dilution of the unknown solution until an approximate match to the standard is obtained. The dilution factor is then introduced into the calculation. This method is obviously not adapted to routine laboratory procedure.

Since the deviation of the observed from the actual values for given concentrations of glucose is constant, it has been found possible to correct the values obtained by the original Folin-Wu method by reference to a curve constructed from readings of solutions of known concentration.

Determinations were conducted on solutions of pure glucose (99.6 per cent by polarimetric and gravimetric copper reduction)



methods) in 0.2 per cent benzoic acid. The colorimetric ratios were plotted as abscissæ against the actual concentrations as ordinates (Fig. 1).

Theoretically the points should fall on the 45° line, but actually they fall on a line of more gentle and gradually diminishing slope. For practical purposes however, and within the limits indicated in Fig. 1, the slight curvature may be disregarded without intro-

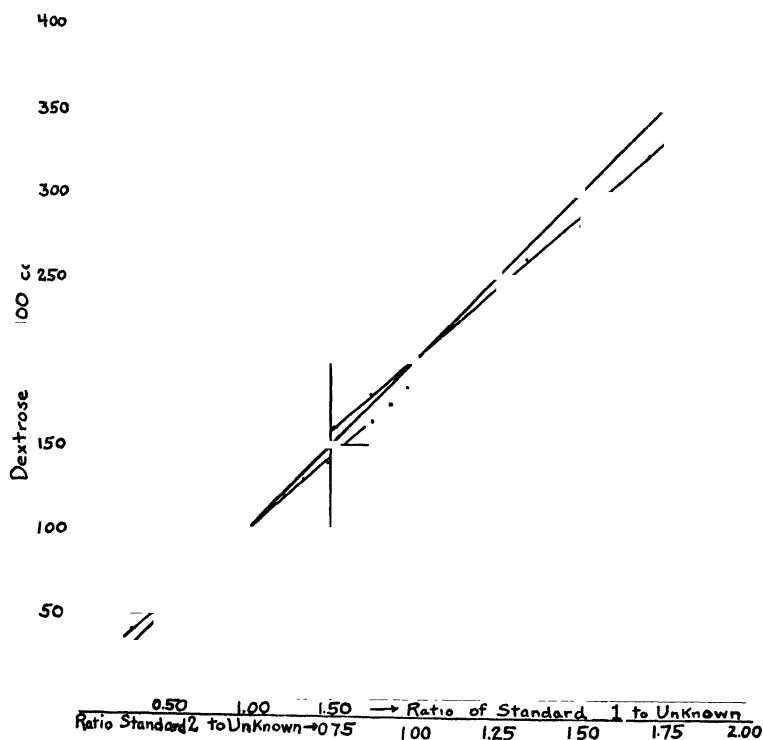


FIG. 1.

ducing an error greater than the error of the method. Assuming then that the values fall along straight lines, the equations for these lines are

$$y_1 = 0.88 x_1 + 12$$

and

$$y_2 = 0.855 x_2 + 20$$

in which  $x_1$  and  $x_2$  are the values obtained when using the weak and strong standards respectively, and  $y_1$  and  $y_2$  are the corresponding actual concentrations.

TABLE I.  
*Blood Sugar Determinations Using Standard 1 (2 Cc. = 0.2 Mg.).*

Colorimetric reading.		Ratio standard unknown	Blood sugar.	
Standard (unknown set at 20).	Unknown (standard set at 20).		Observed ( $x_1$ ).	Actual ( $y_1$ ).
(1)	(2)	(3)	(4)	(5)
<i>mm.</i>	<i>mm.</i>		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
6	66.6	0.30	30	38.4
7	57.1	0.35	35	42.8
8	50.0	0.40	40	47.2
9	44.4	0.45	45	51.6
10	40.0	0.50	50	56.0
11	36.3	0.55	55	60.4
12	33.3	0.60	60	64.8
13	30.8	0.65	65	69.2
14	28.6	0.70	70	73.6
15	26.7	0.75	75	78.0
16	25.0	0.80	80	82.4
17	23.5	0.85	85	86.8
18	22.2	0.90	90	91.2
19	21.1	0.95	95	95.6
20	20.0	1.00	100	100.0
21	19.1	1.05	105	104.4
22	18.2	1.10	110	108.8
23	17.4	1.15	115	113.2
24	16.7	1.20	120	117.6
25	16.0	1.25	125	122.0
26	15.4	1.30	130	126.4
27	14.8	1.35	135	130.8
28	14.3	1.40	140	135.2
29	13.8	1.45	145	139.6
30	13.3	1.50	150	144.0
31	12.9	1.55	155	148.4
32	12.5	1.60	160	152.8
33	12.1	1.65	165	157.2

Values have been calculated from these equations and are recorded in Tables I and II. It may be remarked that the experimental observations upon which these tables are based did not show as great differences from the theoretic values as those reported

by Rothberg and Evans, especially toward the limits of each standard.

Column 1 of these tables gives the readings of the standards when the unknown solutions are set as described by Stoner (3),

TABLE II.

*Blood Sugar Determinations Using Standard 2 (2 Cc. = 0.4 Mg.).*

Colorimetric reading.		Ratio $\frac{\text{standard}}{\text{unknown}}$	Blood sugar.	
Standard (unknown set at 10).	Unknown (standard set at 20).		Observed ( $x_1$ ).	Actual ( $y_2$ ).
(1)	(2)	(3)	(4)	(5)
mm.	mm.		mg. per 100 cc.	mg. per 100 cc.
7.5	26.7	0.75	150	157.3
8.0	25.0	0.80	160	165.8
8.5	23.5	0.85	170	174.4
9.0	22.2	0.90	180	182.9
9.5	21.1	0.95	190	191.5
10.0	20.0	1.00	200	200.0
10.5	19.1	1.05	210	208.6
11.0	18.2	1.10	220	217.1
11.5	17.4	1.15	230	225.7
12.0	16.7	1.20	240	234.2
12.5	16.0	1.25	250	242.8
13.0	15.4	1.30	260	251.3
13.5	14.8	1.35	270	259.9
14.0	14.3	1.40	280	268.4
14.5	13.8	1.45	290	277.0
15.0	13.3	1.50	300	285.5
15.5	12.9	1.55	310	294.1
16.0	12.5	1.60	320	302.6
16.5	12.1	1.65	330	311.2
17.0	11.8	1.70	340	319.7
17.5	11.4	1.75	350	328.3
18.0	11.1	1.80	360	336.8
18.5	10.8	1.85	370	345.4
19.0	10.5	1.90	380	353.9
19.5	10.3	1.95	390	362.5
20.0	10.0	2.00	400	371.0

which procedure eliminates all but a simple mental calculation and is practiced in these laboratories. Column 2 represents the readings obtained when the standards are set as directed by Folin and Wu. In the third column are given the ratios of the readings of

the standards to the readings of the unknowns. The fourth column contains the glucose concentrations in mg. per 100 cc. obtained in the usual way from these readings, while the last column gives the actual glucose concentrations to which these correspond. For glucose concentrations higher than those recorded in Table II, the determinations should be repeated using 1 cc. of filtrate.

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## ON THE NITROGENOUS COMPONENTS OF YEAST NUCLEIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, November 7, 1925.)

It is firmly established that complete hydrolysis of yeast nucleic acids yields four nitrogenous derivatives. On partial hydrolysis, depending upon the temperature and time of hydrolysis, either four nucleosides or four nucleotides are obtained. On the basis of these findings the tetranucleotide theory of the structure of nucleic acid was formulated. From time to time, however, doubts were expressed as to the primary origin of the uracil compound. For a long time Steudel refuted the tetranucleotide structure, but in a more recent publication, in cooperation with Izumi<sup>1</sup> he reported analytical data which, in his opinion, agreed better with the tetra than with the trinucleotide theory.

Very recently, Walter Jones, in cooperation with M. E. Perkins,<sup>2</sup> hydrolyzed yeast nucleic acid in a solution containing an excess of 1 per cent of sodium hydroxide at the temperature of boiling water. Under such conditions, they failed to isolate uridinephosphoric acid from the product of hydrolysis. This result led them to abandon the view of the tetranucleotide structure of yeast nucleic acid. Uridinephosphoric acid was isolated when the nucleic acid was dissolved in a 5 per cent solution of ammonia water and the solution heated for 2 hours at 100°C. In the opinion of W. Jones these conditions suffice to hydrolyze off the amino group from the cytosine complex of the nucleic acid.

Theoretically, it is not easy to conceive that under such mild conditions as hydrolysis with very dilute ammonia, the amino

<sup>1</sup> Steudel, H., and Izumi, S., *Z. physiol. Chem.*, 1923, cxxxi, 159.

<sup>2</sup> Jones, W., and Perkins, M. E., *J. Biol. Chem.*, 1924-25, lxii, 557.

group could be removed from the cytosine complex. There is also another argument which speaks against the view of Jones. Animal nucleic acid has a structure very closely resembling that of plant nucleic acid. Among its nitrogenous constituents, one is cytosine. When the two acids are hydrolyzed under identical conditions, the former yields only an insignificant quantity of uracil, whereas the yeast nucleic acid yields equimolecular proportions of cytosine and uracil and each one of these bases is obtained in equimolecular proportions with the purine derivatives.

However, the point raised by Jones is of sufficient importance to be subjected to a further experimental test. In an easy and decisive way the theory could be tested if, instead of nucleic, cytidinephosphoric acid were used for the experiment. If the view of Jones is correct, cytidinephosphoric acid should yield uridinephosphoric under the conditions under which nucleic acid forms the latter nucleotide.

This test has now been made and the result was negative. The specific rotation of cytidinephosphoric in 5 per cent ammonia water is  $[\alpha]_D^{20} = +44.0^\circ$ , that of uridinephosphoric acid  $[\alpha]_D^{20} = +14.0^\circ$ . Thus, it is easy to detect the change polarimetrically.

The original rotation of cytidinephosphoric acid remained unchanged after 2 and after 3 hours of heating in a sealed tube at  $100^\circ\text{C}$ . From the reaction product unchanged cytidinephosphoric acid could be crystallized with a 50 per cent yield of the original material. In a parallel experiment cytidinephosphoric acid was dissolved in a 5 per cent ammonia solution and from this unheated solution cytidinephosphoric acid was obtained in crystalline form also with a yield of 50 per cent of the original material. Thus, for the present, there seem to be no sufficient grounds to alter the older view as to the tetranucleotide structure of yeast nucleic acid.

#### EXPERIMENTAL.

The cytidinephosphoric acid used in the experiment was perhaps slightly contaminated with adenylic acid. It contained 13.5 per cent nitrogen, whereas the theory requires 13.00 per cent. The specific rotation of the substance was  $[\alpha]_D^{20} = +31.0^\circ$ , whereas the pure substance has a  $[\alpha]_D^{20} = +44^\circ$ . The specific rotation of uridinephosphoric acid in the identical solvent is  $+14.0^\circ$ . 0.300

gm. of cytidinephosphoric was heated in a sealed tube at 100° for 2 hours. The rotations were:

Before heating.	After heating.
$[\alpha]_D^{20} = \frac{+1.89^\circ \times 100}{2 \times 3} = +31.5^\circ$	$[\alpha]_D^{20} = \frac{+1.89^\circ \times 100}{2 \times 3} = +31.5^\circ$

In a second experiment 0.300 gm. of the substance was dissolved as in the previous experiment. The heating was continued 3 hours. The rotations were as follows:

Before heating.	After heating.
$[\alpha]_D^{20} = \frac{+1.87^\circ \times 100}{2 \times 3} = +31.0^\circ$	$[\alpha]_D^{20} = \frac{+1.87^\circ \times 100}{2 \times 3} = +31.0^\circ$

2.0 gm. of cytidinephosphoric acid were dissolved in 20.0 cc. of 5 per cent ammonia water and the solution heated at 100°C. for 2 hours. The solution was then concentrated nearly to dryness. The residue was dissolved in water and the aqueous solution was made strongly acid by means of acetic acid. An equal volume of methyl alcohol was added and the solution was allowed to stand overnight. The crystalline material was filtered off and dried under diminished pressure over sulfuric acid, first at room temperature and later at 55°C. The yield was 1.0 gm. and the substance contained 13.5 per cent nitrogen.

1.0 gm. of cytidinephosphoric acid was dissolved in 10 cc. of 5 per cent ammonia water. The solution was acidified by means of glacial acetic acid and an equal volume of methyl alcohol was added. The following day a crystalline deposit formed. After drying it weighed 0.5 gm.





## THE CONVERSION OF OPTICALLY ACTIVE LACTIC ACID TO THE CORRESPONDING PROPYLENE GLYCOL.

BY P. A. LEVENE AND H. L. HALLER.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, November 17, 1925.)

A previous communication<sup>1</sup> dealt with the conversion of levo- $\beta$ -oxybutyric acid into levo-propylene glycol through the intermediary step of levo-1-amino-2-hydroxypropane. Independently and simultaneously, identical results were reported by P. Karrer and W. Klarer.<sup>2</sup> At the time of our first publication we overlooked, to our regret, an earlier publication by E. Abderhalden and E. Eichwald<sup>3</sup> who, starting from dextro-1-amino-2-chloropropane arrived at levo- $\beta$ -hydroxybutyric acid. According to Abderhalden, dextro-propylene glycol leads to levo- $\beta$ -hydroxybutyric acid. In our experiments as well as in those of Karrer and his coworker,  $\beta$ -hydroxybutyric acid and the propylene glycol obtained from it rotated in the same direction. The causes of the discrepancy are now under investigation.

The present communication contains a report on the conversion of levo-lactic acid into levo-propylene glycol. The propylene glycol was obtained on reduction of the ester of lactic acid by means of metallic sodium by the method of Bouveault as modified in this laboratory. Efforts at converting esters of  $\alpha$ -hydroxy acids into the corresponding glycols according to the original method were not successful. Under the procedures followed in our laboratory the reduction in the desired direction did take place although the yield of the glycol was very small. The product analyzed correctly for propylene glycol in one instance; in another, the substance contained a higher percentage of carbon than is required

<sup>1</sup> Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxx, 49.

<sup>2</sup> Karrer, P., and Klarer, W., *Helv. Chim. Acta*, 1925, viii, 393.

<sup>3</sup> Abderhalden, E., and Eichwald, E., *Ber. chem. Ges.*, 1918, li, 1312.

by theory for the glycol. However, the glycol was readily identified in the form of its diurethane derivative. The diurethane rotated to the right, that is, in the opposite direction from the glycol, which rotated to the left. We have reason to believe that the impurity which was present in a small amount in the glycol was optically inactive, since the higher boiling fraction of the reduction product contained a very high percentage of carbon ( $C = 64$  per cent) and was optically inactive. If our observations and those of Karrer and his coworker regarding the direction of rotation of  $\beta$ -hydroxybutyric acid and of propylene glycol are correct, then dextro- $\beta$ -hydroxybutyric acid and dextro-lactic acid are configurationally related. This conclusion would be in harmony with the fact that both in dextro- $\beta$ -hydroxybutyric acid and dextro-lactic acid the salts have a lower dextro rotation than the corresponding free acids. Further work is now in progress.

#### EXPERIMENTAL.

In the preliminary experiments, a lactic acid of low activity was employed. In a 2 dm. tube the syrup (sp. gr. 1.2) had a rotation of  $-1.15^\circ$ . This syrup was diluted to six times its volume with water and boiled under a reflux condenser for 6 hours. The solution after cooling had the following rotation.

$$[\alpha]_D^{20} = \frac{+0.20^\circ \times 100}{2 \times 18} = +0.55^\circ$$

The acid was converted into the ethyl ester, the rotation of which without solvent was  $-2.53^\circ$  (sp. gr. = 1.035,  $l = 1$ ).

$$[\alpha]_D^{20} = -2.5^\circ$$

The ester, reduced in the manner described below, yielded a propylene glycol having the following rotation in absolute alcohol.

$$[\alpha]_D^{20} = \frac{+0.08^\circ \times 100}{1 \times 12.2} = +0.6^\circ$$

It had the following composition.

0.1376 gm. substance :	0.2408 gm. $CO_2$ and 0.1312 gm. $H_2O$ .
$C_3H_8O_3$ .	Calculated. C 47.37, H 10.52.
	Found. " 47.72, " 10.66.

*Levo-Lactic Acid*.—This acid was prepared by the procedure described by Irvine.<sup>4</sup> The morphine salt was twice recrystallized from 50 per cent alcohol. It was then dissolved in water and decomposed with a slight excess of ammonia. The morphine was filtered off and the filtrate converted into the calcium salt. A sample of the anhydrous salt gave the following rotation.

$$[\alpha]_D^{25} = \frac{+0.21^\circ \times 100}{1 \times 2.7} = +7.8^\circ$$

*Dextro-Ethyl Lactate*.—25 gm. of thoroughly dried calcium lactate were suspended in 150 cc. of absolute alcohol and 12 gm. of concentrated sulfuric acid slowly dropped in, the mixture being thoroughly stirred with a mechanical stirrer. The mixture was heated under a reflux condenser in an oil bath (90–100°) overnight. It was then cooled, the excess sulfuric acid neutralized with potassium carbonate, and the solution filtered from salts. After removal of the alcohol the ester was distilled under diminished pressure. It distilled at 54–56°C.,  $p = 20$  mm. The yield was 14 gm. or 52 per cent of the theory. It analyzed as follows:

0.1012 gm. substance : 0.1856 gm. CO<sub>2</sub> and 0.0774 gm. H<sub>2</sub>O.

C<sub>6</sub>H<sub>10</sub>O<sub>3</sub>.      Calculated. C 50.85, H 8.47.

Found.      " 50.01, " 8.45.

The rotation without solvent was +11.05° (sp. gr. = 1.035,  $l = 1$ ).

$$[\alpha]_D^{25} = +10.7^\circ$$

*Levo-1, 2-Dihydroxypropane*.—Ethyl lactate was reduced in 10 gm. lots with sodium and alcohol in the apparatus described by Levene and Allen.<sup>5</sup> 12 gm. of sodium were suspended in 100 cc. of dry toluene and the mixture was heated. When the sodium was melted the stirrer was rotated vigorously in order to form a fine emulsion. 10 gm. of the ester, dissolved in 25 cc. of alcohol, were then introduced through a dropping funnel at such a rate that there was gentle refluxing. The mixture was continuously

<sup>4</sup> Irvine, J. C., *J. Chem. Soc.*, 1906, lxxxix, 935.

<sup>5</sup> Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 443.

stirred. The addition of the ester required 15 minutes. In order to complete reduction and dissolve all the sodium, absolute alcohol was next introduced. The solution was cooled and 15 cc. of water added. Carbon dioxide was then passed into the solution until it was neutral. The sodium carbonate which settled out was filtered off and thoroughly washed with alcohol and ether. The filtrate and washings were concentrated under diminished pressure. The glycol was obtained from the residue by distillation under diminished pressure. It distilled at 60–70°C.,  $p = 1$  mm. The yield was poor. It analyzed as follows:

0.1007 gm. substance : 0.1838 gm.  $\text{CO}_2$  and 0.0976 gm.  $\text{H}_2\text{O}$ .  
 $\text{C}_3\text{H}_5\text{O}_2$ .      Calculated. C 47.37, H 10.52.  
                     Found.      " 49.77, " 10.84.

The rotation in absolute alcohol was

$$[\alpha]_D^{25} = \frac{-0.65^\circ \times 100}{1 \times 35} = -1.85^\circ$$

*Dextro-Propylene Diphenyl Dicarbamate (Propylene Di- (Phenyl Urethane)).*  $\text{CH}_3\text{CH}(\text{OCONHC}_6\text{H}_5) \text{CH}_2(\text{OCONHC}_6\text{H}_5)$ .—This substance was obtained by heating 1.6 gm. of propylene glycol with 5 gm. of phenylisocyanate under a reflux condenser on the water bath for 2 hours. It was recrystallized three times from 70 per cent alcohol and melted at 136–140°C. It analyzed as follows:

0.0989 gm. substance required 6.30 cc. 0.1 N HCl.  
 $\text{C}_{17}\text{H}_{18}\text{O}_4\text{N}_2$ .      Calculated. N = 8.92 per cent.  
                             Found.      " = 8.91 " "

In absolute alcohol it gave a rotation of

$$[\alpha]_D^{25} = \frac{+0.56^\circ \times 100}{2 \times 10} = +2.8^\circ$$

## THE STRUCTURAL RELATIONSHIP OF THE CARDIAC POISONS.

BY WALTER A. JACOBS AND ALEXANDER HOFFMANN.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, November 23, 1925.)

In former communications investigations have been described which demonstrated definitely the nature of the unsaturated linking in strophanthidin. It was found that this double bond is situated within the lactone ring in all probability between the  $\beta$  and  $\gamma$  carbon atoms so that strophanthidin may be designated as a substituted  $\Delta_{\beta,\gamma}$  crotonic lactone.<sup>1</sup> Characteristic for strophanthidin and all of its derivatives which still possess the double bond of the parent substance is their behavior in dilute pyridine solution towards Tollens' reagent. For example, pseudostrophanthidin, anhydrostrophanthidin and its ethylal, dianhydrostrophanthidin and its ethylal, and the dilactone  $C_{22}H_{28}O_4$ , obtained by oxidation of dianhydrostrophanthidin reduce Tollens' reagent more or less readily. On the other hand the analogous derivatives of dihydrostrophanthidin, in which the olefinic linking has been removed by hydrogenation, no longer react with this reagent or to an extent which is practically negligible in comparison with the behavior of the unsaturated compounds. This is quite striking even in the case of derivatives of dianhydrodihydrostrophanthidin which possess double bonds elsewhere in the molecule than in the lactone ring. Similarly, isostrophanthidin which results from a shift in the double bond of strophanthidin, only after long standing gradually develops a reaction with this reagent. This test has given such concordant results with all of the substances studied that there appears to

<sup>1</sup> Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxiv, 383; 1925, lxxv, 493.

be complete justification in attributing the reduction of Tollens' reagent directly to the unsaturated lactone group.

Results of a similar and most striking character have more recently been obtained by the use of the nitroprusside test. With this reagent strophanthidin in dilute pyridine solution after addition of a few drops of alkali at once gives a deep red color. A similar positive reaction was given by all derivatives of strophanthidin in which the unsaturated lactone group is still present. But as soon as this group was hydrogenated or lost by saponification to the acid the resulting substances no longer gave a positive reaction.

In the course of studies with ouabain, or gratus strophanthin, which had been initiated in this laboratory a number of years ago and which had been temporarily interrupted by the pressure of other work, the unsaturated nature of this substance was ascertained. Ouabain on hydrogenation was found to absorb 1 mol of hydrogen with the formation of a dihydro derivative.<sup>2</sup> These experiments will be described more fully in a communication which will shortly appear. The early work of Arnaud<sup>3</sup> had shown that the glucoside is converted into an acid, ouabaic acid, by the action of hot alkali, a fact which demonstrates its lactone nature. Ouabain is thus unsaturated and at the same time a lactone. Because of the experience with strophanthidin it was of interest to study the behavior of ouabain and its dihydro derivative towards Tollens' reagent and sodium nitroprusside. A striking difference in the behaviors of the two substances was at once observed which was exactly analogous to the contrast noted in the behaviors of strophanthidin and its hydro derivative. Ouabain reduces Tollen's reagent fairly readily and gives a positive nitroprusside reaction, while its hydrogenation product failed to react with these reagents. Similarly after saponification, ouabain no longer gave a color reaction with sodium nitroprusside. It appears therefore strongly indicated that we are dealing with phenomena analogous to those observed with strophanthidin and its derivatives and that ouabain

<sup>2</sup> In the preliminary note (Jacobs, W. A., and Hoffman, W., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxiii, 214) the statement that ouabain is hydrogenated with the formation of a tetrahydro compound should be corrected as given in this paper.

<sup>3</sup> Arnaud, M., *Compt. rend. Acad.*, 1898, cxxvi, 1280.

like this substance contains an unsaturated lactone group. The fact that ouabain absorbs 1 mol of hydrogen on hydrogenation is of interest from another standpoint. If we accept the formula of this glucoside derived by Arnaud,  $C_{30}H_{46}O_{12}$ ,<sup>4</sup> which Thoms<sup>5</sup> has substantiated and which our own analyses have confirmed, this substance is a rhamnoside of an aglucone,  $C_{24}H_{36}O_8$ . Since it is unsaturated and contains no carbonyl group, the saturated hydrocarbon of reference is  $C_{24}H_{42}$ . Since this differs from a paraffin hydrocarbon  $C_{24}H_{50}$  by eight hydrogen atoms it can be only tetracyclic. The ouabain aglucone, although a  $C_{24}$  derivative, would appear therefore to belong to the tetracyclic group of  $C_{24}$  compounds which contains the digitalis aglucones and bufotalin. Strophanthidin, although a tetracyclic compound, is a  $C_{23}$  derivative.

The suggestive character of the ouabain tests have caused us to turn to other substances of this pharmacological group, which have been shown to be lactones. As a result of the recent very important contributions of Windaus<sup>6</sup> and his coworkers, the digitalin verum of digitalis seeds which had been the subject of much previous study by Kiliani, and the sparingly soluble anhydrodigitalin of Kraft<sup>7</sup> (gitoxin of Windaus and Schwarte) obtained from the leaves have been shown to be in all likelihood glucosides of the same aglucone, gitoxigenin,  $C_{24}H_{36}O_5$ . This substance was found to be a lactone and also to contain a double bond. We have prepared gitoxin from digitalis leaves and have studied its behavior towards both Tollens' reagent and sodium nitroprusside. In both cases definitely positive reactions were obtained. After gentle saponification with alkali gitoxin no longer gave a positive nitroprusside reaction. Here again there is a strong indication that gitoxigenin like strophanthidin possesses an unsaturated lactone group.

Still another substance which merited similar study is the easily crystallized and soluble glucoside of digitalis leaves, digitoxin, of which the aglucone, digitoxigenin, has been shown by Kiliani to

<sup>4</sup> Arnaud, M., *Compt. rend. Acad.*, 1888, cvi, 1013.

<sup>5</sup> Thoms, H., *Ber. pharm. Ges.*, 1904, xiv, 114.

<sup>6</sup> Windaus, A., and Bandte, G., *Ber. chem. Ges.*, 1923, lvi, 2001. Windaus, A., Bohne, A., and Schwieger, A., *Ber. chem. Ges.*, 1924, lvii, 1386. Windaus, A., and Schwarte, G., *Ber. chem. Ges.*, 1925, lviii, 1515.

<sup>7</sup> Kraft, F., *Arch. Pharm.*, 1912, ccl, 118.



be also a lactone. This worker, however, was unsuccessful in attempts to hydrogenate this substance.<sup>8</sup> From the recent very careful studies of Cloetta<sup>9</sup> the formula of digitoxigenin is in all probability  $C_{24}H_{36}O_4$ . If this formula is accepted and if also the view is adopted that digitoxigenin is a tetracyclic dihydroxylactone, a simple calculation makes it apparent that digitoxigenin must be derived from a hydrocarbon,  $C_{24}H_{40}$ , which if tetracyclic must contain one double bond. Digitoxigenin would then appear to be a lactone with an unsaturated group.<sup>10</sup> This conclusion was supported by the behavior of the substance towards Tollens' reagent and sodium nitroprusside. Here again positive reactions were obtained and after saponification with alkali a positive reaction with nitroprusside was no longer obtained.<sup>11</sup> It would appear that digitoxigenin also possesses an unsaturated lactone group.

A summary of the results of these observations is briefly given in the accompanying tables. The tests were made as follows: About 10 to 20 mg. of the substance were dissolved in 1 cc. of pure pyridine and diluted with an equal volume of water. The solution was treated with 0.5 cc. of Tollens' reagent and then allowed to stand at room temperature. The reaction occurred gradually as a rule. Definite deposition of silver was apparent during the first 30 minutes at a rate which varied greatly with the individual substance. Those substances which are recorded with negative reactions did not show an appreciable deposit of silver after several hours. In the case of the nitroprusside test a few drops of 10 per cent sodium hydroxide solution were added to the dilute pyridine solution followed by 1 cc. of 0.3 per cent sodium nitroprusside solution. If a positive reaction was given this was shown

<sup>8</sup> Kiliani, H., *Ber. chem. Ges.*, 1918, li, 1631.

<sup>9</sup> Cloetta, M., *Arch. exp. Path. u. Pharmacol.*, 1920, lxxxviii, 133.

<sup>10</sup> Since this communication had been sent to press the last paper of Windaus and Freese (*Ber. chem. Ges.*, 1925, lviii, 2503) has appeared in which the presence of one double bond in digitoxigenin was demonstrated directly by hydrogenation experiments.

<sup>11</sup> In the case of ouabain, gitoxin, and digitoxin the apparent association of the double bond and the lactone group was made even more certain by the fact that if the solutions obtained by saponification with alkali, which no longer gave a positive nitroprusside test, were reacidified and heated to permit relactonization, such solutions again gave a positive nitroprusside test.

TABLE I.  
*Reaction with Tollens' Reagent.*

Strophanthidin.....	+
Dihydrostrophanthidin.....	-
Dianhydrostrophanthidin.....	+
Ethylal of oxidodianhydrostrophanthidin.....	+
"    "    oxidodianhydrostrophanthidinic acid.	+
"    "    dianhydrodihydrostrophanthidin.....	-
Dianhydrodilactone, $C_{23}H_{26}O_4$ .*.....	+
Dianhydrodiacid, $C_{23}H_{30}O_6$ .†.....	+
Tetrahydrodianhydrodilactone, $C_{23}H_{30}O_4$ .†.....	+
Hexahydrodianhydrodilactone, $C_{23}H_{32}O_4$ .‡.....	-
Pseudostrophanthidin.....	+
Isostrophanthidin.....	-
Ouabain.....	+
Dihydroouabain.....	-
Gitoxin.....	+
Digitoxin.....	+
Bufagin.....	+

\* Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxx, 501.

† Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxx, 503.

‡ Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1925, lxx, 504.

TABLE II.  
*Reactions with Sodium Nitroprusside.*

Strophanthidin.....	+
Dihydrostrophanthidin.....	-
Dianhydrostrophanthidin.....	+
Dianhydrostrophanthidinic acid.....	-
Ethylal of oxidodianhydrostrophanthidin.....	+
"    "    oxidodianhydrostrophanthidinic acid.	-
"    "    dianhydrodihydrostrophanthidin.....	-
Dianhydrodilactone, $C_{23}H_{26}O_4$ .....	+
Dianhydrodiacid, $C_{23}H_{30}O_6$ .....	-
Tetrahydrodianhydrodilactone, $C_{23}H_{30}O_4$ .....	+
Hexahydrodianhydrodilactone, $C_{23}H_{32}O_4$ .....	-
Pseudostrophanthidin.....	+
Isostrophanthidin.....	-
Oubain.....	+
"    saponified.....	-
Dihydroouabain.....	-
Gitoxin.....	+
"    saponified....	-
Digitoxin.....	+
"    saponified..	-
Bufagin.....	-

at once by the appearance of a deep red color. When negative a yellow color only was observed.

It will be of interest to extend these studies to other "genins" belonging to this general group of cardiac poisons, such as anti-arigenin, scillarigenin, bufotalin, and the like. Through the kindness of Dr. J. J. Abel we have already obtained a small sample of bufagin, the poison isolated by Abel and Macht<sup>12</sup> from the tropical toad, *Bufo agua*. This substance was found to reduce Tollens' reagent very promptly, but in its behavior towards sodium nitroprusside it proved to be an exception to the rule. But the significance of this failure to give a positive nitroprusside test cannot be evaluated in view of our very meager knowledge of the chemistry of the substance.

Of very great importance in connection with the results here reported are the interesting observations of Windaus, Bohne, and Schwieger<sup>13</sup> with regard to the influence of hydrogenation on the toxicity of digitalin. Whereas 0.5 mg. of digitalinum verum proved to be a lethal dose for a 35 gm. frog, 6 and 8 mg. of the dihydro derivative proved to be non-toxic. We have made a few crude similar toxicity tests on frogs to compare the effect of hydrogenation on ouabain. Whereas 0.02 mg. of ouabain proved to be a lethal dose, frogs of about 40 gm. weight just tolerated doses of 2 mg. or 100 times as much dihydroouabain. It is not excluded that the amorphous dihydro compound which we have employed may still have contained traces of unchanged ouabain. At any rate these experiments suggest that the unsaturated group of these compounds may be essential for their pharmacodynamic effect. It will be of interest to confirm this by the further study of the effect of hydrogenation on the toxicity of other substances of this group.

Although the observations which have been reported here may not be construed as conclusive evidence, nevertheless they are strongly suggestive of the probability that the aglucones of ouabain, of the digitalis glucosides and perhaps of other substances of this pharmacological group, possess, like strophanthidin an unsaturated

<sup>12</sup> Abel, J. J., and Macht, D. I., *J. Pharmacol. and Exp. Therap.*, 1911-12, iii, 334.

<sup>13</sup> Windaus, A., Bohne, A., and Schwieger, A., *Ber. chem. Ges.*, 1924, lvii, 1388.

lactone group and that this group may be essential, perhaps in conjunction with other structural features, for the pharmacodynamic action of these substances. We are at present attempting to ascertain by more direct chemical methods, as has already been accomplished in the case of strophanthidin, whether these substances are indeed inner esters of enolized ketones. An attempt will also be made to confirm if possible the suggested responsibility of this unsaturated lactone group for the cardiac action of this group of poisons by the synthesis and pharmacological study of simpler substances containing unsaturated lactone groups.



## NOTES ON THE METABOLISM OF AMINO AND FATTY ACIDS.

By H. D. DAKIN.

(*Sarborough-on-Hudson.*)

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The following communication contains short notes on a variety of experiments dealing with the general subject of amino and fatty acid catabolism. While the material is of a rather fragmentary character and in some cases not susceptible of rigid interpretation it is possible that it contains certain points of interest to those engaged in this line of work and hence may justify publication.

### *I. The Fate of $\gamma$ -Triphenylpropionic Acid in the Animal Body.*

While Knoop's theory of the  $\beta$ -oxidation of normal fatty acids is based on such a variety of evidence that its general truth can hardly be questioned (1), there is of course no good reason for assuming that initial oxidation of hydrogen in the  $\beta$ -position is the only type of initial transformation of saturated normal fatty acids that the body is capable of effecting. The stability in the animal body of certain acids such as phenylacetic acid which, since  $\beta$ -oxidation is precluded, might but do not undergo  $\alpha$ -oxidation may conceivably be due to their ready coupling with glycine or glutamine. The resulting conjugated compounds, as is well known, are very resistant to further change. The possibility of the oxidation of normal saturated fatty acids in other than the  $\beta$ -position has been emphasized by the recent careful work of Clutterbuck and Raper (2) in which they show conclusively that salts of normal fatty acids when oxidized with hydrogen peroxide undergo oxidation at the  $\gamma$  and  $\delta$  carbon atoms as well as at the  $\beta$ - and possibly also at the  $\alpha$ -position.

It was therefore of interest to investigate the fate in the animal body of an acid in which  $\beta$ -oxidation for structural reasons could

not occur, in order to see if  $\alpha$ -oxidation might then be observed. The substance chosen was  $\beta$ -triphenylpropionic acid,  $(C_6H_5)_3 \cdot C \cdot CH_2 \cdot COOH$ , which was obtained by the hydrolysis of triphenylmethylmalonate obtained by the condensation of triphenylchloromethane and sodium malonic ester. The synthesis using triphenylmethylbromide instead of the more accessible chloride has been described in detail by Henderson (3).

The sodium salt of the acid (2 gm.) was given by mouth and also subcutaneously to rabbits. Absorption is somewhat slow so that the urine was collected for a 48 hour period. On acidifying the urine with sulfuric acid and shaking with ether two or three times a large amount of oily acid was readily recovered. Apart from the usual small amounts of aromatic hydroxy acids found in rabbit's urine, practically the whole of the ether extract was made up of unchanged triphenylpropionic acid which was readily isolated in the form of its very sparingly soluble barium salt. On analysis the barium salt was found to contain 18.3 per cent Ba compared with a calculated value of 18.5 per cent. The barium salt on decomposition with hydrochloric acid gave the free acid which after recrystallization from formic acid melted at  $175-177^\circ$ . It also gave a crystalline phenylhydrazine salt sparingly soluble in alcohol and almost insoluble in ether, melting at  $147-148^\circ$ . The yield of recovered triphenylpropionic acid varied from 55 to 72 per cent of that administered. The urine after removal of the unchanged acid was carefully examined by the customary methods for other products but apart from a normal amount of hippuric acid no other crystalline aromatic acid could be detected. No indications were obtained of any glycine or other derivative of triphenylpropionic acid or of triphenylacetic acid.

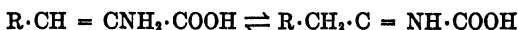
It would appear therefore that under the conditions of the experiments outlined, triphenylpropionic acid, a substance in which  $\beta$ -oxidation cannot occur but in which, for structural reasons,  $\alpha$ -oxidation is not precluded, is largely excreted unchanged. The failure to undergo oxidation cannot be ascribed to conjugation with glycine or allied substances since this does not occur in the rabbit. The experiments therefore furnish no evidence indicating the possibility of initial oxidation of fatty acids in the  $\alpha$ -position.

It has occurred to the writer that an alternative hypothesis may, at least in certain cases, be worthy of consideration. It is rather a striking fact that with the single exception of glycine, which in some respects appears to show an anomalous behavior in the animal body, all the biologically important amino acids contain at least one unsubstituted hydrogen atom in the  $\beta$ -position. Usually two such hydrogen atoms are present except in the cases of the  $\alpha$ -amino- $\beta$ -hydroxy acids or their sulfur analogues. Assuming the possibility that  $\alpha$ -amino acids might undergo  $\beta$ -oxidation of the type well established for normal fatty acids, one would obtain an unsaturated amino acid of the type,  $R \cdot CH = CNH_2 \cdot COOH$ . Now substances of this character are notoriously unstable and most reactions in which they would be expected to be formed yield instead  $\alpha$ -ketonic acids and ammonia. For example the hydrolysis of benzoyl  $\alpha$ -aminocinnamic acid gives phenylpyruvic acid. Furthermore, these  $\alpha$ -amino- $\alpha\beta$ -unsaturated acids



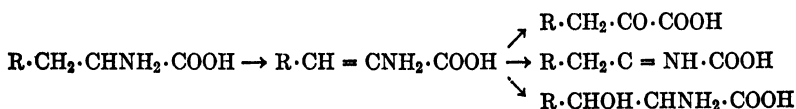
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are isomeric with the imino acids regarded by Knoop as initial products of amino acid oxidation and in at least two cases, namely the esters of aminomethylene malonic acid and of aminofumaric acid, there is evidence of their mutual convertibility by tautomeric change:



If  $\alpha\beta$ -unsaturated  $\alpha$ -amino acids should prove to be initial products of the oxidation of amino acids it would follow that: (a) The initial stages of the oxidation of amino and normal fatty acids would be essentially similar in type and possibly have certain mechanisms in common. (b) The formation of  $\alpha$ -ketonic acids *in vivo* from  $\alpha$ -amino acids would be easily accounted for. (c) The formation of amino acids such as serine,  $\beta$ -hydroxyglutamic acid and cysteine from the corresponding unsaturated amino acids would be readily comprehensible and correlated with the known formation of malic acid from fumaric acid *in vivo*.

The hypothetical changes which an  $\alpha$ -amino acid might undergo in accordance with the ideas outlined above may be represented as follows:



The experimental testing of the possible formation of  $\alpha\beta$ -unsaturated  $\alpha$ -amino acids as initial products of the oxidation of  $\alpha$ -amino acids is a matter of some difficulty. As a first attempt it was decided to investigate the behavior in the body of  $\alpha$ -amino acids containing no hydrogen attached to the  $\beta$ -carbon atom and hence incapable of undergoing normal  $\beta$ -oxidation. Such acids would be expected to be more resistant to oxidation in the body than the corresponding acids containing hydrogen in the  $\beta$ -position, if indeed,  $\alpha\beta$ -dehydrogenation should prove to be a normal reaction of amino acids. Accordingly various attempts were made to synthesize acids of the type  $R^1R^2R^3C \cdot CHNH_2 \cdot COOH$ , particularly those in which at least one R is represented by a phenyl group and hence more easily followed in its metabolism. Unfortunately triphenylalanine could not be obtained by the usual synthetic methods nor could  $\beta$ -phenyl- $\beta$ -dimethylalanine be

obtained starting from acetophenone and converting it into phenyldimethylchlormethane. The condensation of the latter substance with malonic ester proceeded abnormally and the required acid could not be obtained for subsequent bromination and treatment with ammonia. Up to the present the only  $\beta$ -trisubstituted amino acid available is the  $\alpha$ -amino- $\beta$ -trimethylpropionic acid already described by Knoop and Landmann (5) but whose fate in the body appears not to have been investigated.<sup>1</sup> This substance is obviously not ideal for the purposes in view since it is known that methyl groups may be rather readily removed from acids with branched chains. In the absence of more suitable material it seemed worth while to investigate the fate of  $\beta$ -trimethyl- $\alpha$ -aminopropionic acid in the animal body.

2 gm. portions of the amino acid in aqueous solution were injected subcutaneously into rabbits of 1.5 to 2 kilos weight. The urine was collected for 48 hours and then examined by the method described below. In four experiments the following quantities of optically inactive unchanged amino acid were recovered from the urine: 0.60, 0.72, 1.05, and 1.16 gm. or an average of about 46 per cent of the substance given. Under similar circumstances the administration of the isomeric leucine does not lead to more than about 10 per cent excretion of unchanged acid. It would therefore appear that the absence of any unsaturated hydrogen in the  $\beta$ -position of trimethyl- $\alpha$ -aminopropionic acid may be related with its relatively difficult combustion in the body, but when the fact is recalled that this particular isomer of leucine is not a normal metabolic product, it would seem rash to draw any far reaching conclusion from the present experiments. The possibility of the formation of  $\alpha\beta$ -unsaturated amino acids by the oxidation *in vivo* of normal amino acids must be regarded at present as nothing more than a somewhat plausible hypothesis.

The rabbit urines were examined in the following manner. Urea was first of all removed by the action of urease. The filtered residue was then rendered acid with sulfuric acid and concentrated to small volume. Hippuric and other acids were then removed by

<sup>1</sup> In describing the preparation of the amino acid, Knoop has apparently overlooked the fact that the ester of the acid as well as the oxime of trimethylpyruvic acid had already been described by Richard, A., *Ann. chim. et phys.*, 1910, series 8, xxi, 323.

thorough extraction with ether in a continuous extractor. The aqueous residue, after extraction, was neutralized with sodium carbonate and then gently warmed on the water bath with a couple of gm. of potassium cyanate, so as to convert any amino acid into the ether-soluble uramino acid. On subsequent acidification and extraction with ether  $\alpha$ -uramino- $\beta$ -trimethylpropionic acid was readily obtained and purified by crystallization from water. It was weighed, its purity controlled by its melting point, and then converted for further identification into the corresponding hydantoin by boiling with a little dilute acid. The uramino acid and hydantoin obtained from the urine were optically inactive and were identical in every respect with these substances prepared directly from the pure amino acid.

*$\alpha$ -Uramino- $\beta$ -trimethylpropionic acid* was prepared in almost theoretical yield by warming the corresponding amino acid in aqueous solution with its own weight of potassium cyanate. On acidifying, the uramino acid at once crystallizes out. It was purified by recrystallization from boiling water and separates usually as hexagonal plates but occasionally in needles. It melts sharply at  $221^{\circ}$  (uncorrected) with effervescence. It is very sparingly soluble in cold water and in ether but readily soluble in alcohol.

*Analysis.* 0.1472 gm. substance: 0.2617 gm.  $\text{CO}_2$  and 0.1086 gm.  $\text{H}_2\text{O}$ .  
 $\text{C}_7\text{H}_{14}\text{O}_3\text{N}_2$ . Calculated. C 48.3, H 8.04  
 Found. " 48.5, " 8.20

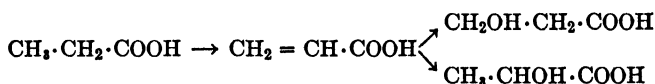
*Tert-butylhydantoin* was easily obtained by boiling the preceding uramino acid for 15 minutes with 10 per cent hydrochloric acid. The substance readily crystallizes from boiling water in stout hexagonal prisms melting without decomposition at  $242$ – $243^{\circ}$  (uncorrected). It is sparingly soluble in cold water and in ether but readily soluble in alcohol.

*Analysis.* 0.1460 gm. substance: 9.2885 gm.  $\text{CO}_2$  and 0.0842 gm.  $\text{H}_2\text{O}$ .  
 $\text{C}_7\text{H}_{12}\text{O}_3\text{N}_2$ . Calculated. C 53.8, H 7.70  
 Found. " 53.9, " 7.85

### III. The Action of Muscle and Liver Tissue on Acrylic and Hydracrylic Acids.

The question as to the mode of oxidation of propionic acid in the animal organism is a puzzling one. Ringer (6) has shown that

propionic acid, like lactic acid, yields glucose approximately quantitatively in the phlorhizinized dog, thus suggesting that the propionic acid might undergo  $\alpha$ -oxidation to lactic acid. Blum and Woringer (7) have stated that they have detected both pyruvic acid and lactic acid in the urine of animals receiving propionic acid though it must be confessed that the evidence adduced as regards pyruvic acid seems inconclusive and Knoop is inclined to question the interpretation of these observations since many other structurally unrelated acids tend to promote the excretion of lactic acid. The direct conversion of propionic into lactic acid would furnish a unique example of  $\alpha$ -oxidation of a normal fatty acid and hence would be of considerable interest. But an alternative hypothesis may be put forward by which propionic acid might undergo dehydrogenation of the  $\alpha\beta$ -type yielding acrylic acid which in turn might yield hydracrylic or conceivably lactic acids. Furthermore it should be noted that acrylic acid, like propionic acid, yields glucose in the phlorhizinized animal. The changes that propionic acid might undergo according to this scheme may be represented as follows:



The formation of acrylic acid from propionic acid would be analogous to the known oxidation of succinic acid to fumaric acid by muscle and the formation of hydracrylic or lactic acids would be analogous to the formation of malic from fumaric acid, a balanced reaction which is also readily brought about by muscle tissue (8). It was therefore of interest to determine whether muscle or liver tissue could effect any similar changes with the three carbon acids. The results were entirely negative, and no evidence was obtained of the formation of either hydracrylic or lactic acids from acrylic acid, neither was there any conversion of hydracrylic into acrylic acid. The experiments therefore throw no new light on the problem of the mechanism of the oxidation of propionic acid.

Acrylic acid and hydracrylic acid were both prepared from  $\beta$ -chloropropionic acid according to well known methods. The digestions with fresh tissue and the sodium salts of the acids were carried out in the same fashion and with the same concentrations

as those described in a previous paper (8). Appropriate controls were carried out simultaneously. The following method was devised for the estimation of acrylic acid and gave thoroughly satisfactory results. A portion of the filtrate (usually 0.5 cc.) after coagulation of proteins by heat was mixed with 10 cc. of 5 per cent sodium carbonate solution. A known excess of decinormal potassium permanganate was then added and after 30 seconds excess of potassium iodide and sulfuric acid were added. The liberated iodine was a measure of the unreduced permanganate and was titrated with standard sodium thiosulfate. Under these conditions a molecule of acrylic acid takes up about six atoms of oxygen while hydracrylic acid takes up none. The solutions are readily standardized with acrylic acid of known strength. In no case, using rabbit and chicken muscle and liver, was any disappearance of acrylic acid noted nor was any unsaturated acid formed from hydracrylic acid.

#### *IV. The Fate of Some Uramino Acids and Hydantoins in the Animal Body.*

While it is known that benzoyl and some other acyl derivatives of amino acids are excreted unchanged when fed to animals, not much is known of the behavior of uramino acids and the hydantoins derived from them. The question is of some importance in connection with Werner's (9) views of the formation of cyanic acid as an obligatory product of the oxidation of amino acids since if this were true one might expect the formation of uramino acids *in vivo*. Miss Rohde (10) has shown that the uramino acid derived from leucine is partly excreted unchanged on intravenous infusion into anesthetized animals. In the present experiments all the substances were given as neutral sodium salts by stomach tube to rabbits—usually doses of 1 gm. per kilo using 2 kilo rabbits were employed. The results may be summarized as follows:

$\alpha$ -Uramino-isobutylacetic acid and the corresponding isobutylhydantoin prepared from *dl*-leucine were both excreted unchanged to the extent of at least 72 to 95 per cent. The same result was obtained with  $\alpha$ -uraminophenylpropionic acid and benzylhydantoin prepared from *dl*-phenylalanine. There was no evidence of any nuclear hydroxylation analogous to the formation

of tyrosine from phenylalanine described by Embden as occurring in the liver. The excreted products were all optically inactive. The foregoing products were all isolated by ether extraction and purified by crystallization from water. Small amounts of hippuric acid which could be removed by hydrolysis were the only other crystalline substance obtained. It may fairly be inferred that the preceding substances are all virtually resistant to oxidation in the body and since they are not found in normal urine it is fairly certain that they are not produced in ordinary metabolism.

Markedly different results were obtained with glutamic and aspartic acid derivatives. Uraminoglutaric acid (5 gm.) prepared from *D*-glutamic acid (11) was given by mouth to a rabbit (2.5 kilos) as the sodium salt. Only about 10 per cent could be accounted for in the urine. Practically an identical result was obtained with the corresponding hydantoinpropionic acid after giving 3 gm. Hydantoinacetic acid (3 gm.) prepared from *L*-aspartic acid (11) under similar conditions was excreted to the extent of 20 to 36 per cent in two experiments. It is clear therefore that the uramino and hydantoin derivatives of aspartic and glutamic acids are mainly oxidized in the animal body thus differing from the similar derivatives of the monamino acids. This difference, it would appear, must be referable to the presence of the acetic and propionic side chains present in the former substances, and the results may be regarded as indirect evidence in favor of the idea that glutamic acid itself may undergo oxidation in other positions than at the  $\alpha$ -carbon atom carrying the amino group.

The examination of the urines obtained in the foregoing experiments was conducted according to the following outline. The 48 hour specimens were collected and urea removed with urease, which does not attack the substances under investigation. Phosphates were removed with calcium hydroxide and the filtrate concentrated. It was then made just acid to Congo red with sulfuric acid and allowed to stand. Hippuric acid and calcium sulfate were filtered off and the filtrate extracted with ether to remove volatile acids, etc. The aqueous residue was freed from chlorides with silver nitrate and the uramino or hydantoin derivatives were then precipitated as silver salts in neutral solution or with basic lead acetate. The silver or lead salts were decomposed

with hydrogen sulfide and the substances identified and estimated by polarization and direct crystallization. None of the uraminoglutaric acid could be recovered in the crystalline form but the syrup on warming with dilute hydrochloric acid readily gave hydantoinpropionic acid which crystallizes more readily. The other substances were readily obtained crystalline and identified. It may be added that in a single experiment in which optically inactive hydantoinacetic acid was fed, the product excreted was dextrorotatory and hence contained an excess of the form derived from *d*-aspartic acid, *i.e.*, the optical antipode of the *L*-aspartic acid derived from proteins.

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## THE PREPARATION OF *d*-ARGININE CARBONATE.

By ARNOLD E. PRATT.

(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor.)

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In the course of the preparation of a considerable quantity of arginine for use in experimental work, certain difficulties were experienced with the method of Kossel and Gross (1). The procedure outlined by these authors depends upon the precipitation of arginine from a protein hydrolysate with 2,4-dinitro-1-naphthol-7-sulfonic acid, followed by a decomposition of this arginine salt and formation of arginine carbonate. The protein was first hydrolyzed with 33 per cent by volume sulfuric acid and after removal of the sulfuric acid with barium hydroxide, the arginine was precipitated by the addition of dinitronaphthol-sulfonic acid. In this laboratory it was found that apparently a large amount of the arginine was adsorbed by the great bulk of barium sulfate precipitate, even after thorough washing, since the amount of arginine dinitronaphtholsulfonate formed was only about 55 to 60 per cent of the theoretical as calculated from the arginine content of the protein.

After digestion with hot water and washing of the precipitated arginine salt to remove soluble impurities, Kossel and Gross decomposed it by heating with 33 per cent by volume sulfuric acid. Upon cooling, the dinitronaphtholsulfonic acid precipitated and the arginine remained in the sulfuric acid solution. On account of the fact that the precipitation of the dinitronaphtholsulfonic acid was not complete, the portion left in solution was removed with decolorizing carbon. Since a fairly large amount of this was necessary, a loss of arginine may have resulted from adsorption by the carbon. After removal of the dinitronaphtholsulfonic acid it was again necessary to precipitate the sulfuric acid in the solution with barium hydroxide. Here also the opportunities



for the loss of arginine in adsorption by the barium sulfate were large. An added disadvantage of the method was the difficulty in filtering and washing so bulky a precipitate. After removal of the excess barium hydroxide with carbon dioxide, the precipitate of barium carbonate was filtered off, the filtrate concentrated to a small volume, and the arginine carbonate allowed to crystallize. Kossel and Gross further recommend that this crude arginine carbonate be converted into the picrate which may be recrystallized and converted into the carbonate in the usual manner. The total yield of crude arginine carbonate from gelatin by this method was found, in this laboratory, to be only about 25 per cent of the theoretical.

In an attempt to improve upon the method of Kossel and Gross in the yield and purity of arginine carbonate as well as in the facility of laboratory manipulation, the following facts were noted. (1) Arginine dinitronaphtholsulfonate is easily soluble in hot dilute sulfuric acid, containing 5 cc. of the concentrated acid (sp. gr. 1.84) per 100 cc., and nearly insoluble in cold acid of this strength. Hence, this solvent is ideal for the recrystallization of the salt. (2) Dinitronaphtholsulfonic acid is readily soluble in *n*-butyl alcohol and is easily extracted by it from an aqueous solution containing a little sulfuric acid. Since Dakin (2) has demonstrated that arginine is not extracted by butyl alcohol, this served very well for the extraction of the dinitronaphtholsulfonic acid from a dilute sulfuric acid solution of its arginine salt.

If, as in the procedure of Kossel and Gross, the arginine dinitronaphtholsulfonate which is used for the preparation of arginine carbonate is not pure, a subsequent purification of the arginine becomes necessary. Due to the great solubility of arginine carbonate a direct purification of this substance by recrystallization is difficult. This makes necessary a conversion into a more insoluble salt (Kossel and Gross suggest the picrate), which may be obtained pure by recrystallization and then reconverted into pure arginine carbonate. The advantages of recrystallizing the arginine dinitronaphtholsulfonate before its decomposition are obvious. It was found that arginine carbonate prepared from the recrystallized dinitronaphtholsulfonate was of such a degree of purity that a repurification was unnecessary.

For the hydrolysis of the protein, concentrated hydrochloric

acid (sp. gr. 1.18) was used since the excess of acid after hydrolysis could easily be removed by distillation *in vacuo* and the tedious process of removing the sulfuric acid with barium hydroxide, together with the accompanying losses in arginine, avoided.

The details of the method as finally adopted are as follows: 500 gm. of protein (*e.g.*, gelatin) are boiled for 18 hours with 1 liter of concentrated hydrochloric acid. After the hydrolysis is complete, the excess of hydrochloric acid is removed as completely as possible by distillation *in vacuo*, on the steam bath. The thick gummy residue of the hydrochlorides of the amino acids is next dissolved in 1 liter of hot distilled water; the hot solution is treated with a small amount of a good grade of decolorizing carbon and filtered. The arginine is then precipitated by adding to the hot hydrolysate, a hot concentrated aqueous solution of 2, 4-dinitro-1-naphthol-7-sulfonic acid, containing 4 parts by weight of the acid to 1 part by weight of the arginine expected. Upon cooling, the arginine dinitronaphtholsulfonate will precipitate out. The flask is allowed to stand 24 hours in the ice box and the precipitate is then filtered off with suction and washed with a small amount of ice cold water on the funnel. The salt is now recrystallized from dilute sulfuric acid containing 50 cc. of the concentrated acid (sp. gr. 1.84) per liter. About 2 liters will be necessary. After standing in the ice box for 24 hours to complete the precipitation, the arginine dinitronaphtholsulfonate, which crystallizes out in beautiful golden yellow plates, is filtered off with suction and thoroughly washed on the funnel with small quantities of ice cold water. After as much as possible of the wash water has been removed by suction, the salt is dried in the vacuum desiccator. The yield of pure arginine dinitronaphtholsulfonate should be almost quantitative since the loss in recrystallization is small.

The arginine dinitronaphtholsulfonate is best decomposed in quantities of 25 gm. or less. The salt is dissolved in hot dilute sulfuric acid of the same concentration as that used for the recrystallization. The hot solution is then extracted with *n*-butyl alcohol in the usual manner in a separatory funnel. During the first few extractions the solution must be kept sufficiently hot to prevent the precipitation of the arginine dinitronaphtholsulfonate from the aqueous layer. If this occurs, the arginine salt will tend to collect at the interface between the two layers, and thus

prevent a clear-cut separation of the two solutions. Moreover, if the arginine dinitronaphtholsulfonate is not entirely in solution, there is a considerable tendency for small particles of the salt to pass into the butyl alcohol layer in the form of a colloidal suspension. The extractions are continued until the aqueous layer is colorless, or nearly so. The butyl alcohol extracts are then combined and extracted once with a small amount of water, to remove any arginine which may be in the butyl alcohol extracts, and then this aqueous extract is extracted with butyl alcohol until colorless, or nearly so, as before. The aqueous solutions, which contain all of the arginine as the sulfate and free from dinitronaphtholsulfonic acid, are combined. The sulfuric acid is now precipitated with a saturated solution of barium hydroxide in slight excess of the theoretical amount required to neutralize the sulfuric acid. The barium hydroxide solution must be added slowly with constant stirring to insure a coarsely grained precipitate which will settle out well and be easy to filter and wash. The end-point may be determined by removing a drop from the solution and placing it in a depression on a spot plate containing a dilute alcoholic solution of phenolphthalein as an indicator. An excess of barium hydroxide is indicated by a strongly alkaline reaction. It must be pointed out that a good quality of barium hydroxide, reasonably free from hydroxides of alkali metals, must be used since the carbonates of these metals will appear as impurities in the final arginine carbonate.

Carbon dioxide is now passed into the solution containing the barium sulfate precipitate until it is saturated. This precipitates the excess of barium hydroxide and converts the arginine into the carbonate. The precipitate of barium sulfate and carbonate should settle out and after standing overnight to complete the precipitation, it may be easily filtered off. The precipitate is washed once by decantation, and several times on the funnel with cold water.

The filtrate and washings are then combined. If they are not water-clear they must be warmed on the steam bath with a small amount of decolorizing carbon and filtered. The solution should be slightly alkaline due to the presence of arginine carbonate. It is now transferred to a large evaporating dish and concentrated to a volume of 75 to 100 cc. If at this point a small precipitate of

inorganic salts appears, it must be filtered off. This is unlikely if sufficiently pure barium hydroxide is used. After transferring to a small crystallizing dish the evaporation is continued on the steam bath, until the solution is concentrated to a thin syrup. After cooling, crystallization is started by scratching the bottom of the dish with a glass rod. The syrup will completely solidify in the form of microscopic oblong plates. The arginine carbonate is dried *in vacuo*. The yield on the basis of protein used is about 85 or 90 per cent theoretical. In this way 35 to 40 gm. of arginine carbonate may be prepared from 500 gm. of gelatin.

The absence of other bases forming insoluble or slightly soluble picrolonates was proved by the preparation of a picrolonate from arginine carbonate obtained as above. The carbonate was dissolved in the smallest possible amount of water and picronic acid in alcoholic solution, in slight excess of the theoretical amount, was added with stirring. The arginine picrolonate, which precipitated in the form of fine crystals, was filtered off, and after thorough washing with absolute alcohol, dried *in vacuo*. Without recrystallization, the picrolonate melted at 237.5° (uncorrected), with decomposition, in an open tube. The bath was heated to 220° before insertion of the melting-point tube. The temperature was taken with an Anschütz thermometer. Picrolonates prepared from three commercial samples of arginine, from different sources, melted at the same point. This is 6.5° higher than the melting point, 231°, reported by Riesser (3) and 12.5° higher than the figure 225° given by Steudel (4) and accepted by Weiss (5). The picrolonate contained one molecule of water of crystallization which was lost by drying at 110°, as reported by Riesser (3).

The 2,4-dinitro-1-naphthol-7-sulfonic acid used in this method may be obtained commercially as the free acid, or it may be prepared from the dye naphthol yellow S which is an alkali salt of this acid. The dye is dissolved in hot 33 per cent sulfuric acid and upon cooling, the free acid precipitates out. It is filtered off and recrystallized from water. The melting point is 151° (uncorrected).

The butyl alcohol may be recovered from the butyl alcohol extracts by distillation. For recovery of the dinitronaphthol-sulfonic acid, the residue, after the butyl alcohol has been removed by distillation, is added to the original mother liquors from the

first precipitation of the arginine, which contain considerable quantities of this acid, and the dinitronaphtholsulfonic acid precipitated by the addition of half the volume of concentrated sulfuric acid. The precipitate is filtered off and recrystallized from water as above.

The author wishes to take this opportunity to express his gratitude to Prof. Howard B. Lewis of this laboratory for his many helpful suggestions during the progress of this work.

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## THE DETERMINATION OF SUGAR IN BLOOD AND IN NORMAL URINE.

By OTTO FOLIN.

(*From the Biochemical Laboratory of Harvard Medical School, Boston.*)

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In a recent paper, S. R. Benedict<sup>1</sup> describes a colorimetric copper method for the determination of the blood sugar which is based upon the idea that his well known copper citrate reagent should prove as superior for quantitative work on blood as it has been for qualitative tests on urine. This expectation seems to have been fulfilled to a certain extent, for by a series of parallel blood sugar determinations he shows that the new method does give materially lower values than are obtained by the method of Folin and Wu.

Benedict seems to believe that the only essential feature of his qualitative sugar reagent is the fact that its alkali is a carbonate instead of a hydroxide. This concept I think is not quite correct. When Benedict, in 1908,<sup>2</sup> described his citrate reagent the most important new feature was the use of citrate instead of a tartrate, and the purpose, as stated by Benedict, was to obtain a more stable reagent than had been obtained by the use of Rochelle salt. Increased stability was, however, not the only result, or indeed the most important result achieved by substituting citrate for tartrate. The citrate exerts a powerful depressive action on the oxidative properties of dissolved copper compounds and it is because of this inhibiting effect that the original citrate reagent proved so suitable as a qualitative reagent for sugar in urine. The inhibiting effect of the citrate includes the reaction with reducing sugars, and it is because of this factor that citrate reagents are qualitatively less sensitive and quantitatively give much less cuprous oxide from a given amount of sugar (0.2 to 0.4 mg.)

<sup>1</sup> Benedict, S. R., *J. Biol. Chem.*, 1925, lxiv, 207.

<sup>2</sup> Benedict, S. R., *J. Biol. Chem.*, 1908-09, v, 485.

than do corresponding reagents prepared by the help of tartrates. The antireducing effect of citrates is most conveniently demonstrated by introducing one or two drops of a 20 per cent sodium citrate solution to a Folin-Wu sugar tube and comparing the color obtained from the standard glucose solution in this tube with that obtained without any added citrate—by the Folin-Wu method.

The explanation given above as to the characteristic behavior of alkaline copper citrate solutions toward reducing substances, including glucose, does not agree very well with the theoretical interpretations and arguments which Benedict has advanced in support of his new method, yet neither does it necessarily, or materially, affect the validity of his main working hypothesis, namely, that the process which gives the lowest sugar values with diluted normal urine should yield the most nearly correct values when applied to blood filtrates. My own explanation on the other hand suggests that there should be other and more flexible methods for meeting the requirements of this ingenious hypothesis than through the general antireducing action of the citrates.

The first obvious inquiry in this particular problem was naturally to determine the extent to which it is permissible to reduce the alkalinity of a given copper solution. I have done this with the Folin-Wu reagent and I have thus found that sodium carbonate alone is altogether too strong an alkali with such active copper solutions as are obtained with the help of tartrates. By reducing the alkalinity almost down to the reaction of sodium bicarbonate one still gets just as much cuprous oxide from 0.2 to 0.4 mg. of glucose as with the original Folin-Wu reagent, and when such a weakly alkaline reagent is applied directly to diluted normal human urine, the reduction obtained is very much smaller than that obtained with the original reagent.

The reagent finally adopted is made as follows:

*New Alkaline Copper Tartrate Solution.*—Dissolve 12 gm. of Merck's sodium tartrate (or 15 gm. of Rochelle salt) together with 7 gm. of anhydrous sodium carbonate and 20 gm. of sodium bicarbonate in 600 to 700 cc. of distilled water. Transfer this solution to a volumetric liter flask and to it add 5 gm. of copper sulfate previously dissolved in about 200 cc. of water. Dilute to volume and mix.

In the preparation of this reagent all the carbon dioxide which is

generated when the copper sulfate is added should be retained in the solution by combining with a part of the surplus carbonate. In practice it may therefore be desirable, though scarcely necessary, to insert a stopper in the volumetric flask and shake for a few moments, immediately after the addition of the copper sulfate solution.

When the "sugar" of normal human urine is determined directly on the diluted urines by means of this copper solution and by Benedict's solution, no uniform proportionality is obtained. With some urines Benedict's reagent will give a little less sugar and with others it will give more. This is, of course, what might be expected from such widely different reagents when applied to such complex and varying reducing mixtures as human urines—which may contain no glucose at all. In order not to make such comparisons unduly favorable to the new copper reagent described above the urines should be first rendered just alkaline to phenolphthalein; though the difference in result is insignificant provided that the urines have not been preserved by means of preservatives which are acid in reaction.

Before applying the new copper reagent to actual blood sugar determinations it seemed best also to try to improve the molybdate solution which is used for the development of the blue color by means of which one measures the amount of cuprous oxide obtained in the reduction. That molybdate solution was developed specifically for use in connection with the copper tartrate reagent of Folin and Wu and it is, therefore, not surprising that Benedict found it unsuitable for use in connection with his copper reagent. In resorting to the use of the uric acid reagent for the development of the blue color I think that Benedict has not found a reagent that is particularly suitable. The uric acid reagent will give more color, to be sure, with a given quantity of cuprous copper than does the special phosphate molybdate reagent of Folin and Wu, but the uric acid reagents give almost no color with cuprous copper in mixtures sufficiently acid in reaction to dissolve promptly cuprous oxide. Benedict's reagent, for example, gives extremely little color if added directly to a freshly prepared, dilute, solution of cuprous chloride. The residual acidity obtained when 2 cc. of the uric acid reagent are added to 2 cc. of his copper reagent is so low that the mixture will develop a blue color with uric acid. Also,



and for the same reason, the blank, that is to say, the blue color obtained on merely mixing equal volumes of the two reagents is much greater than the corresponding blank in the Folin-Wu method. The most serious consequence of the low degree of acidity obtaining in the sugar tubes is, however, the fact that the cuprous oxide sediment dissolves only very slowly. Benedict has evidently intended to provide for this flaw in his method by inserting a waiting period of 10 minutes between the addition of the uric acid reagent to the cooled contents in the sugar tube and the dilution with water. It is quite possible that this provision is adequate for most cases, but those who use the process should know that they need to be alert to see whether the cuprous oxide actually has dissolved. If the cuprous oxide is abundant and for one reason or another has settled more completely than usual, all the cuprous oxide may not dissolve even in an hour. That this is so can easily be demonstrated by heating 0.4 mg. of glucose for 20 minutes before cooling and then adding the uric acid reagent. I tried this experiment in order to see whether the yield of cuprous oxide could be increased, but was unable to determine the point, because of the insolubility of the cuprous oxide. (In order to see the undissolved cuprous oxide within the dark blue solution, close inspection against a black background is necessary.)

I had two objects in mind in trying to improve upon the phosphate-molybdate reagent of Folin and Wu. First, to try to secure a deeper color from a given amount of cuprous oxide and, second, to increase the acidity so that it could be used with almost any alkaline copper reagent. Both of these objects have been attained, though only at the expense of considerable effort. The new molybdate solution also gives more dependable proportionality between different amounts of cuprous oxide. When the standard is set at 20 mm., readings of the unknown between 10 mm. and 40 mm. are perfectly dependable.

*New Acid Molybdate Reagent for the Estimation of Cuprous Copper.*—The sodium molybdate at present obtainable is not very pure. It contains some insoluble material which it is best, though not strictly necessary, to remove by filtration. In addition, the molybdate contains some reducing impurity which gradually imparts a blue color to the finished reagent unless it is destroyed

by oxidation (with bromine). If the reagent is wanted for immediate use or for a short time, 3 or 4 days, one can ignore these impurities, and then the preparation of the reagent takes only a few minutes. For such purposes the preparation is made as follows:

Dissolve 40 gm. of sodium molybdate,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , in 100 cc. of distilled water, in a 500 cc. beaker. To this somewhat turbid solution add, with stirring, 55 cc. of 85 per cent phosphoric acid, 40 cc. of 25 per cent sulfuric acid, and 20 cc. of 99 per cent acetic acid—in the order given. (The sulfuric acid is obtained by adding one volume of concentrated acid to three volumes of water and cooling.) The resulting mixture is at once ready for use.

For the preparation of larger quantities of the reagent for permanent use, the process is as follows:

Dissolve 150 gm. of sodium molybdate,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , in 300 cc. of distilled water. Filter through a 15 cm. quantitative filter paper into a flask (capacity 1 liter) and wash with 75 cc. of water. To the sodium molybdate solution in the flask add several drops (0.1 to 0.2 cc.) of bromine and shake for a few minutes till the bromine has dissolved. Let stand for an hour to complete the oxidations produced by the hypobromite. Then add, with shaking, 225 cc. of 85 per cent phosphoric acid. The surplus bromine is set free and imparts a yellow color to the solution. After all the phosphoric acid has been added, add also 150 cc. of cooled 25 per cent sulfuric acid (one volume of concentrated sulfuric acid added to three volumes of water). Remove the surplus bromine by means of a moderately rapid air current. The aeration will take about half an hour. Finally, add 75 cc. of 99 per cent acetic acid, mix, and dilute to a volume of 1 liter.

If it is inconvenient to remove the bromine immediately after the addition of the sulfuric acid, let the mixture stand, covered with a beaker, till the following day. Such extra standing with bromine is just as good. The reducing impurities can in fact be destroyed just as well by adding the bromine to the acidified solutions, but 3 to 4 days standing is then required to complete the process, and it is for this reason that the hypobromite oxidation was tried. This molybdate solution is used exactly as the molybdate solution of Folin and Wu; but when used with Benedict's copper citrate

reagent it is necessary to use 4 cc. instead of 2 cc. The solution is practically colorless, and will remain so if kept free from organic impurities.

Critical readers will be apt to wonder whether there is any good reason for using both sulfuric acid and acetic acid as well as an abundance of phosphoric acid in the preparation of this molybdate reagent. To this I can only say that the given combination of acids represents the outcome of a large number of experiments and as the preparation is easy and the reagent good, I have not considered it worth while to try to secure further simplification. I suspect that the active compound in this reagent is a phosphomolybdic acid not yet isolated or described.

In taking up the application of the new solutions for quantitative sugar determinations the reader is reminded that in the new copper reagent we are dealing with a far more delicately adjusted solution than any heretofore used for such purposes. The acidity of the sugar solution or blood filtrate whose sugar content is to be determined is, therefore, a matter of importance. A small surplus alkalinity makes no appreciable difference, because it is taken care of by the bicarbonate; but, as a general rule, it may be stated that the solution should be nearly neutral.

The blood filtrates obtained according to the protein precipitation process of Folin and Wu are nearly neutral when the reagents used are properly adjusted. 10 cc. of a blood filtrate on titration with tenth normal alkali and phenolphthalein give an end-point with about 0.2 cc., indicating an acidity of only about 0.05 normal. As there is no guarantee that all are getting such practically neutral blood filtrates it seems best to require that the blood filtrate be neutralized before it is used for the sugar determination. This is most conveniently done as follows:

Transfer 2 cc. of blood filtrate to a test-tube; add one drop of phenolphthalein solution and count the number of drops of tenth normal alkali required to give the pink end-point. Then simply add the same number of drops of alkali to the sugar tube before introducing the blood filtrates. As the blood filtrates in any one laboratory and with any one set of precipitating reagents will run very uniform, the addition of the alkali to the sugar tube need not always be preceded by the actual preliminary titration. If

the conditions are right there is in fact no need for any neutralization. These precautions are mentioned because it is to be feared that some are still using more or less acid tungstates for the precipitation of the blood proteins. Blood filtrates obtained from blood which has been preserved with sodium fluoride are also apt to be strongly acid, evidently because of the presence of acid impurities in this salt.

*Standard Glucose Solution.*—The concentrated glucose solution containing 10 mg. of glucose per cc. is made up, as before, with nearly saturated benzoic acid solution as the solvent and preservative. But in making up the dilute solutions, containing 0.1 and 0.2 mg. per cc., it is not permissible to dilute 1 and 2 cc. of the strong sugar solution with benzoic acid solution, because to do so would be to introduce an unnecessary complication because of the resulting acidity. For use with the new method the dilution is made with water and a few drops of formalin or toluene are added as an additional preservative.

The sugar determination on the Folin-Wu blood filtrates are made as follows:

Transfer 2 cc. of standard sugar solution to one of two Folin-Wu sugar tubes and transfer 2 cc. of the nearly neutral or neutralized blood filtrate to another similar tube. Add 2 cc. of the new copper solution to each and heat in a boiling water bath for 10 minutes. Cool in a beaker of water for 1 minute or as much longer as may be convenient. Then add 2 cc. of the new special acid molybdate reagent to each test-tube. The cuprous oxide in the tubes dissolves practically instantly and as soon as the visible evolution of  $\text{CO}_2$  has nearly ceased (about 1 minute), dilute to the 25 cc. mark, mix, and make the color comparison.

It will be noted that in the directions given above, the special blood sugar tube of Folin and Wu is still recommended. In his latest paper Benedict affirms that the blood sugar tube of Folin and Wu can be replaced by an ordinary open test-tube by simply replacing most of the air in the tube with an inert gas or vapor (benzene) and keeping the mouth of the test-tube closed with a cotton plug. I was rather surprised over this conclusion of Benedict's for I had tried many such devices in the research which led to the Folin-Wu tube. I now believe that Benedict's conclusion is based on compensating experimental errors. These errors in

brief are due to the use of his uric acid reagent for the development of the blue color. The facts seem to be that in the constricted sugar tube the uric acid reagent dissolves the cuprous oxide more slowly than in open test-tubes, so slowly in fact that much of the uric acid reagent is decomposed and made useless before all the cuprous oxide has dissolved. The loss of color thus sustained counterbalances the loss of cuprous oxide by reoxidation in the open tubes. By using the new molybdate reagent described in this paper (4 cc.) together with Benedict's process I find that the substitute arrangement suggested by Benedict does not appreciably prevent the reoxidation of cuprous oxide.

It was only after the preparation of the two reagents described above had been worked out that I began to check up the analytical findings reported in Benedict's paper. When I did begin to make blood sugar determinations I did so with the three different copper solutions; that is to say with the new copper solution, with the

TABLE I.  
*Mg. Glucose per 100 Cc. of Blood.*

Benedict.....	80	100	98	123	137
Folin-Wu.....	84	103	108	119	140

solution of Folin and Wu, and with Benedict's. The new acid molybdate solution was used only with the first two copper solutions, for even when 4 cc. are taken it is not entirely satisfactory with Benedict's copper reagent, because of its huge citrate contents.

In the course of these parallel determinations it was found that Benedict's method did not at all give the low values reported by Benedict; on the contrary, it would give the same and in some cases even higher blood sugar values than those obtained by the copper reagent of Folin and Wu. The last five sugar determinations which were made on blood taken from normal persons (students) gave the figures in Table I.

The citrate-sulfite solution employed in these determinations was about 2 months old. Benedict states that his copper reagent keeps for a year, but this statement obviously cannot be entirely correct. The sulfite in the reagent must necessarily be subject to spontaneous oxidation to sulfate. The most practical way of

demonstrating the deterioration of the reagent is to prepare a duplicate reagent without any added sulfite and then compare these reagents from time to time by the amount of cuprous oxide which they yield with the standard sugar solution.

Benedict incorporated 0.1 per cent sodium bisulfite in his copper reagent in order to increase the yield of cuprous oxide and it certainly does in a large measure make up for the loss produced by the use of citrates. In the course of my efforts to discover why Benedict's method in my hands did not yield less blood sugar than the Folin-Wu copper reagent, it occurred to me that the disappearance of the sulfite from my 2 months old citrate reagent might have something to do with it. I therefore prepared two new copper citrate reagents, one of which did not contain any added

TABLE II.  
*Mg. Sugar per 100 Cc. of Blood.*

Benedict 1.....	174	151	116	218	230	292
“ 2.....	131	121	89	180	184	242
Difference.....	43	30	27	38	46	50

TABLE III.  
*Mg. Glucose per 100 Cc. of Blood.*

Folin-Wu.....	152	320	296	516	333
Benedict.....	120	277	246	448	304
Difference.....	32	43	50	68	29

sulfite. A series of blood sugar determinations was then made according to Benedict's directions by means of these two copper solutions. The figures in Table II were obtained.

It will be seen that the first row of figures obtained by the citrate reagent to which no bisulfite had been added is much higher than those obtained with the freshly prepared regular Benedict reagent. It should also be pointed out that these blood filtrates represent diabetics with high blood sugar. From figures given in Benedict's paper one finds that he also obtained relatively just about as large differences in blood sugar content by his method and the Folin-Wu method in diabetic bloods with high glucose content as was found in the blood from persons who did not carry a high blood sugar.

His figures are given in Table III.

The blood sugar values obtained by means of Benedict's copper solution only a few days old are the same or a little higher than those obtained with the new copper tartrate solution (see Table IV).

From Benedict's figures (Table III) one must reach the somewhat astonishing conclusion that the non-glucose products in blood capable of reducing alkaline copper solutions are much more abundant in bloods with abnormally high sugar levels than in bloods of normal persons. If such a conclusion should turn out to be correct, then it obviously becomes important to determine the non-glucose reduction as well as the glucose content of blood, particularly diabetic blood. And no one sugar method can meet the requirements of this new situation.

The figures given in Table V will serve to indicate the different sugar values obtained by the new and the Folin-Wu copper reagents in blood filtrates whose sugar levels are within the normal limits.

In Table VI are given the corresponding blood sugar figures obtained from diabetic bloods by means of the two copper reagents.

All of these blood filtrates were obtained from Dr. Joslin's diabetic clinic at the Deaconess' Hospital. No attempt was made to correlate the findings with the insulin or dietetic treatment which the patients were receiving. From the figures given in Table VI it is clear that all diabetic bloods do not behave in exactly the same way. In most cases the sugar values obtained by the two copper reagents are rather farther apart than in the case of bloods from non-diabetic persons; but this is not invariably so, and at the present time it is not possible to go beyond the bare statement that all diabetic bloods do not behave alike.

In the determination of sugar in normal urine according to the method of Folin and Berglund<sup>3</sup> one obtains strikingly different results on substituting the new copper reagent for the old one. Many of the reducing carbohydrate materials found in normal human urines have comparatively little effect on the new copper reagent and one obtains correspondingly less "sugar" in such urines. These findings are in harmony with the idea expressed by

<sup>3</sup> Folin, O., and Berglund, H., *J. Biol. Chem.*, 1922, li, 209.

TABLE IV.

*Mg. Sugar per 100 Cc. of Blood.*

Benedict.....	262	200	118	87	100	124
Folin.....	266	190	116	82	91	109
Difference.....	4	10	2	5	9	15

TABLE V.

*Mg. Sugar per 100 Cc. of Normal Human Blood.*

Folin-Wu.....	130	111	110	118	94	84
Folin.....	127	99	96	101	80	70
Difference.....	3	12	14	17	14	14

TABLE VI.

*Blood Sugar in Mg. per 100 Cc. of Blood.*

Folin-Wu.	Folin.	Difference.
103	77	26
108	87	21
119	89	30
109	84	25
286	266	20
121	116	5
212	190	22
240	210	30
236	198	38
172	160	22
180	180	0
352	352	0
232	212	20
250	224	26
220	195	25
236	216	20
140	131	9
144	127	17
230	204	26
272	248	24
228	218	10
286	272	14
300	260	40
364	352	12
312	280	32



Folin and Berglund that normal human urine contains extremely little, if any, glucose.

At my request, O. Watkins, at the Huntington Hospital, made a series of sugar determinations on urines by means of the two copper reagents. The urines were first treated with Lloyd's alkaloidal reagent as in the Folin-Berglund method. The heating period adopted was 8 minutes with the copper reagent of Folin and

TABLE VII.  
*Mg. of Glucose per 100 Cc. of Urine.*

On the Lloyd filtrate.		On diluted urine.	
Folin-Berglund.	Folin.	Folin.	Benedict.
84	59	112	108
315	155	378	359
209	104	244	239
93	39	118	121
50	29	69	67
89	49	113	114
97	37	115	124
85	36	92	110
79	35	97	95
37	15	49	51
69	38	84	86
116	48	125	165
102	67	112	116
72	51	95	97
9	Trace.	12	11
79	59	76	78
15	10	19	17
74	56	97	82

Wu, and 10 minutes with the copper reagent described in this paper.

The "sugar" was also determined directly on the same urines by Benedict's method and by the new method.

The results obtained are shown in Table VII. The Benedict copper citrate solution used by Watkins was also unfortunately several weeks old and may have lost most of its sulfite.

#### DISCUSSION.

It must be obvious to all that the work described in this paper was produced in response to Benedict's stimulating paper on the

same subject. While I have found that Benedict's concrete new technique is open to serious criticism, the fact remains that he has experimentally demonstrated by that method that the Folin-Wu sugar values are too high—if by blood sugar is meant blood glucose. My own work has in the main confirmed his analytical findings and has also in the main verified the correctness of his working theory that the sugar method which gives the lowest sugar values when applied to urine, if it is at the same time dependable for glucose, will give blood sugar values which correspond most nearly to the glucose content of blood.

Without wishing to detract in the slightest degree from the important service which Benedict has rendered in thus giving a fruitful new turn to blood sugar investigations, I permit myself to quote a passage from my Harvey Lecture of 1919-20 on the same subject:

"In this connection I would call attention to the highly peculiar fact that bloods from nephritics having very high urea retention give by our original method, as by all other sugar methods, abnormally high values for the blood sugar. There does not seem to be any tangible reason why such bloods should contain any more sugar than does the blood of normal individuals. There is room for the suspicion that in such bloods other materials than sugar play an important part, that similar products in smaller amounts are present in all blood, that all sugar values are high and that the lowest sugar values obtained must still be regarded as maximum values."

To this passage something can perhaps be added now:

While a part of the high sugar values obtained in nephritic blood may be represented by reducing products which are not sugar or sugar derivatives, it is not only possible, but highly probable that a very large fraction of these unknown reducing materials is of exactly the same sort as the non-glucose carbohydrate materials which occur in normal urines. These more or less foreign and unusable sugars and sugar derivatives which are eliminated by normal kidneys would naturally accumulate in the blood when the kidneys fail to work. Further, there is no reason to assume that normal kidneys excrete these foreign carbohydrate derivatives except in response to a floating supply of these products in the blood. Nor can one definitely deny that the blood sugar may contain some maltose.

These conclusions are, of course, only another aspect or applica-

tion of Benedict's thesis that reducing substances encountered in the urine must also be present in the blood. This thesis does not imply that the various reducing substances sustain the same quantitative ratio to one another in the blood as in the urine. Creatinine, for example, so abundant in urine, occurs only in negligible quantities in the blood. Every one has probably recognized the theoretical validity of this thesis, but it remained for Benedict to show that it is experimentally demonstrable.

The abnormally high non-glucose reducing materials in diabetic blood would seem to require a different explanation. The non-glucose reducing products in such bloods may in part represent some form of intermediary carbohydrate metabolism. At all events they would seem to merit further investigation.

## DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

### VII. THE INFLUENCE OF SUNLIGHT UPON CALCIUM EQUILIBRIUM IN MILKING COWS.\*

BY E. B. HART, H. STEENBOCK, C. A. ELVEHJEM, AND  
H. SCOTT,

*(From the Department of Agricultural Chemistry, University of Wisconsin,  
Madison.)*

AND G. C. HUMPHREY.

*(From the Department of Animal Husbandry, University of Wisconsin,  
Madison.)*

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In 1923 (1) we published data showing that mixed green grasses added to a ration of corn silage and a grain mixture were unable to maintain a positive lime balance in cows of liberal or even average milking capacity. *These experiments were conducted in a basement room and away from the influence of direct sunlight.* In that paper we said, "It must be remembered that these experiments did not include the possible influence of another potent factor now known to operate in maintaining calcium and phosphorus equilibrium in growing animals; namely, sunlight." In the meantime data have been accumulated and published (2) showing the marked influence of *ultra-violet light* generated from a quartz mercury vapor lamp in maintaining calcium and phosphorus equilibrium in the milking goat.

While these latter results showed very effectively the relation of ultra-violet light to our problem it was believed necessary that further investigations with milking animals and sunlight itself should be undertaken. This was essential for several reasons. There is a growing traffic in the use of minerals, mainly calcium

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carriers, in the feeding of farm animals in this country. The conditions under which rations should be supplemented with calcium salts are not completely known. This is because the factors operating in the assimilation of calcium and phosphorus have not been completely investigated. It is now well known that sunlight is an effective antirachitic agent and in its broadest sense a tendency to a rachitic condition is what is being dealt with in negative lime balance in mature animals.

In all of our work with cows the experiments have been conducted in a basement room away from the influence of direct light. Consequently such results as were obtained may not always apply to animal feeding and especially to summer pasture conditions under which most cows are kept for a part of the year. For example, it is now known (3) that a clover hay of relatively low antirachitic properties would not maintain a positive lime balance in a milking goat kept away from direct sunlight; but such hay with its liberal lime content was completely effective in maintaining a positive lime balance in the same animal when that animal received ultra-violet light generated from a quartz mercury lamp. Possibly the same phenomenon would prevail under the influence of summer sunlight. Consequently, the results we have obtained in our earlier experiments conducted out of access to direct sunlight find their greatest application to winter feeding rather than to summer feeding. While no experiments with farm animals on the effectiveness of winter sunlight in respect to calcium and phosphorus assimilation have as yet been made, nevertheless it is evident from available data on the ultra-violet content of winter sunlight (4) that such light is very ineffective as an antirachitic agent.

#### EXPERIMENTAL.

We planned this experiment as a fair imitation of summer conditions; that is, the provision of green grasses and direct sunlight. On January 1, 1925, six cows (five pure bred Holsteins and one pure bred Ayrshire) were reserved for this work, and from that date until the conclusion of the metabolism work indoors they were not allowed out-of-doors or exposed to direct sunlight. Their premetabolism ration consisted of corn silage, alfalfa hay of unknown curing history, and a grain mixture of yellow corn,

ground oats, wheat bran, and oil meal, fed at the rate of 1 pound for 3 to 4 pounds of milk produced. 1 week before initiation of the metabolism work the alfalfa hay allowed was reduced one-half and timothy hay substituted. This was done in order to reduce the calcium content of the ration to a point approximating the calcium intake of the experimental ration.

On May 4 the metabolism experiment proper was initiated and three of the Holstein cows were used. They had freshened in March and were yielding 45 to 60 pounds of milk daily when placed on the experiment. The plan was to conduct indoors and out of direct sunlight a metabolism experiment of 3 weeks duration and then on the same ration continue the metabolism work, but with the animals in the direct sunlight as much as feasible. For the first half of the experiment—no sunlight—the animals were kept in our permanent metabolism stalls in the basement of the University Dairy Barn. For the second half of the experiment—in sunlight—metabolism stalls were constructed in a large judging room in which the animals could be fed and sheltered at night and to which they could be moved in case of inclement weather. During the day when weather conditions permitted they were removed to stalls which had been constructed out-of-doors and which afforded a maximum exposure to direct sunlight. These stalls were on the southerly exposure of the University Dairy Barn and accomplished what we desired.

During the entire experimental period—6 weeks—the daily ration used consisted of approximately 30 pounds of corn silage, 40 pounds of fresh lawn clippings, and 1 pound of a grain mixture for 3 pounds of milk produced. The grain mixture consisted of 59 parts of yellow corn, 25 parts of wheat bran, 15 parts of oil meal, and 1 part of common salt. Additional common salt was allowed daily. The lawn clippings were composed of June grass (*Poa pratensis*) and white clover (*Trifolium repens*). A soft natural water containing 2 gm. of calcium oxide per 110 pounds was used.

Quantitative collection of all excreta was made by men working in 8 hour shifts and quantitative analysis for calcium of all ingo and outgo recorded. The green plant tissue was sampled daily and analyzed for its lime content. The corn silage was sampled weekly for the same purpose. Phosphorus determinations were

not made because in previous experiments with the same ration positive phosphorus balances had been observed on such a ration as here used, even when the experiment was conducted away from

TABLE I.  
*June Record of Calcium Balance and Milk Production of Cow 1.*

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO ex- creted.	Total CaO intake.	Balance per week.	Milk per week.
Green grass—no-sunlight period.							
	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
May 5-12.....	324.0	20.52	320.3	664.8	474.3	-190.5	405.5
“ 12-19.....	346.8	26.28	308.0	681.1	474.5	-206.6	399.1
“ 19-26.....	360.6	20.88	296.9	678.4	482.5	-195.9	380.2
Green grass—sunlight period.							
	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
May 26-June 2.....	340.2	11.80	302.6	654.6	518.3	-136.3	385.3
June 2-9.....	356.4	15.91	276.9	649.2	514.5	-134.7	373.9
“ 9-16.....	316.8	11.95	287.5	616.3	501.7	-114.6	377.0

TABLE II.  
*June Record of Calcium Balance and Milk Production of Cow 2.*

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO ex- creted.	Total CaO intake.	Balance per week.	Milk per week.
Green grass—no-sunlight period.							
	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
May 5-12.....	315.6	10.96	302.4	629.0	474.3	-154.7	419.0
“ 12-19.....	286.8	7.28	300.1	576.2	474.5	-101.7	413.2
“ 19-26.....	280.2	5.93	294.2	580.3	482.5	-97.8	405.1
Green grass—sunlight period.							
	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
May 26-June 2.....	281.4	6.31	287.2	574.9	518.3	-56.6	400.5
June 2-9.....	270.6	6.10	273.9	550.6	514.5	-36.1	386.7
“ 9-16.....	315.0	5.91	275.7	596.6	501.7	-94.9	389.3

direct sunlight. Under such conditions *negative calcium* balances had prevailed.

In Tables I, II, and III are shown the results of these first experiments conducted out of sunlight and in direct sunlight.

The data are consistent in showing that positive calcium balances could not be maintained in these cows kept out of direct sunlight and with a ration made of grains, corn silage, and green grasses,—comparable, if not superior, to the general summer pasture allowed dairy cattle. A weekly negative balance of 100 to 200 gm. of calcium oxide prevailed in the case of each individual.

With exposure to direct sunlight and consumption of the same ration negative calcium balances still continued. The degree of negativity was, however, reduced. In the case of Cow 1 an average negative lime balance of 197 gm. per week prevailed during the no-sunlight period; while in the sunlight this amount was

TABLE III.  
*June Record of Calcium Balance and Milk Production of Cow 3.*

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Green grass—no-sunlight period.							
	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
May 5-12.....	396.0	3.36	218.9	618.3	462.9	-155.4	319.3
“ 12-19.....	342.6	9.83	221.7	564.1	463.0	-101.1	321.3
“ 19-26.....	336.0	8.11	213.9	548.0	471.0	-77.0	310.0
Green grass—sunlight period.							
	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
May 26-June 2.....	358.8	5.09	216.7	580.6	506.8	-73.8	312.0
June 2-9.....	286.8	4.80	205.6	497.2	502.9	+5.7	301.9
“ 9-16.....	379.2	7.24	209.9	596.3	490.2	-106.1	294.5

reduced to 128 gm. In the case of Cow 2 the weekly average negative lime balance was 118 gm. during the no-sunlight period and 62 gm. during the sunlight period; in the case of Cow 3, 111 gm. during the no-sunlight period and 58 gm. during the sunlight period. It is apparent that calcium equilibrium could not be maintained on the ration used even when the animals were in sunlight.

It should be emphasized that the ration used compared favorably with the summer ration received by most of the dairy cattle in our northern states. It was a ration comparatively low in lime, containing on the air-dried basis about 0.60 per cent of CaO.



What the result would have been had the lime intake been increased either through addition of lime to the ration or through the use of a lime-rich roughage such as clover or alfalfa hay cannot be stated at this time.

These animals were exposed generously to direct sunlight. Daily at 9.00 a.m. they were removed to the outdoor stalls and remained in the sunlight 6 to 7 hours, except in rainy weather. During the 3 weeks in the sunlight period the animals received an exposure to direct sunlight of 109.5 hours or a daily average of 5.2 hours. Since the experiment was conducted from May 25 to June 14,—a period of the year when the “antirachitic region of the solar spectrum” is, according to Hess (5), of greatest intensity and at a time of day when the ultra-violet radiation is at a maximum, it must be granted that the experiment was a fair trial of the optimum natural conditions for maintaining calcium equilibrium in liberally milking cows with the ration used.

The results secured somewhat surprised us. We had anticipated that the sunlight exposure would be more effective in reducing the negative lime balance. In the case of goats exposed  $\frac{1}{2}$  hour daily to the radiations of a quartz mercury lamp the fecal calcium is markedly reduced in the course of 2 weeks (3) as compared with the preexposure period. This was not the case with these cows exposed to sunlight—at least not to any marked degree. It is evident that in respect to the particular problem we were studying, namely the maintenance of a positive lime balance in a milking animal, that the radiations of a quartz mercury vapor lamp were much more effective than solar radiation, apparently at its best.

Since our cows had been exposed to sunlight but 3 weeks there was the possibility that too short a time had elapsed for the development of a maximum power for calcium assimilation. There was the possibility that this power was cumulative and that more time was needed if we desired to imitate the actual sunlight exposure to which dairy cattle are subjected. Consequently it was decided to continue these three animals through the summer on a ration with a lime intake approximately similar to that of the metabolism period and also with maximum sun exposure; further, these cows were to be placed in the metabolism stalls—in sunlight—for a lime balance on September 14 and the metabolism trial was to be continued for 4 weeks. During the time between

June and September the animals were kept in an outdoor paddock during the daytime, which afforded a maximum exposure to sunlight.

In this paddock there was some grass but it constituted but a very small part of the ration. The ration consisted of a grain mixture, corn silage, and a timothy hay. 10 pounds of timothy hay and 30 pounds of silage were allowed daily. 1 pound of the grain mixture for 3 pounds of milk was also fed. Since the timothy hay was of low lime content—0.41 per cent CaO—a daily addition to the ration of each animal of 28 gm. of marl carrying 48.5 per cent of CaO was made. This addition of marl made the lime intake in the ration approximately that of the green grass period used in June. We were forced to displace the green grass ration with the timothy hay because of the probable unavailability of green grasses throughout late summer.

Milk production of all of these animals gradually lowered; No. 1 in June gave approximately 400 pounds per week and in September 140 pounds; No. 2 gave in June 400 pounds per week and in September 250 pounds; No. 3 gave in June 300 pounds and in September 180 pounds. How far such shrinkage in milk production is to be attributed to an insufficient ration, particularly in respect to calcium assimilation, is a proper question to raise. Because of the marked shrinkage in milk production we decided to exclude Cow 1 from the September metabolism trial. Her daily milk production had decreased from over 50 pounds per day in June to 20 to 25 pounds per day, which would make her of less value as a test of the capacity of the ration we were using plus the sunlight environment to maintain a positive lime balance. In her place we substituted a grade Holstein cow that had freshened in August, 1925, and was giving 40 to 45 pounds of milk daily. This cow, called No. 4, had been exposed to sunlight daily throughout the summer and also had received a ration consisting of a grain mixture, corn silage, and timothy hay, fortified with some marl. 2 weeks before being placed in the metabolism trial she was given the same ration as that fed the other two animals—1 pound of a grain mixture for 3 pounds of milk, 30 pounds of corn silage, and 10 pounds of the low lime timothy hay, fortified daily with 28 gm. of marl carrying 48.5 per cent of CaO. The grain mixture con-

sisted of 59 parts of yellow corn, 25 of wheat bran, 15 of oil meal, and 1 part of common salt. This ration was continued throughout the sunlight September metabolism trial for the three animals. It was designed to give a lime intake practically equivalent to that fed in June when the animals received green grasses. As a matter of fact the September ration contained slightly more lime than did the June ration; but the weekly intake in all periods, both June and September, was approximately 500 gm. of CaO.

At the time of the metabolism trial in May and June none of the animals was bred. At the time of the September experiment No. 2 had been bred on July 4, No. 3 on June 2, but No. 4 was not bred.

During the September metabolism trial the cows were treated exactly as they had been in the sunlight period of June. During the night and in cloudy or rainy weather they were kept indoors in the metabolism stalls used in June. During the day and when weather conditions permitted they were removed to the metabolism stalls constructed out-of-doors and which afforded a maximum exposure to sunlight. The experiment was started September 14 and lasted until October 11, 1925. Daily at 9.00 a.m. when weather conditions permitted they were removed to the outdoor stalls and remained in the sunlight 6 to 8 hours. During the early part of the experimental period we were able to secure generous exposure to sunlight but during the last 2 weeks there was considerable cloudy weather. In the 28 days of the experiment there were 7 days when weather conditions prevented removal of the animals to the outdoor stalls. Further, during the 28 days of the experiment the cows received 105 hours of sunshine or an average of 3.7 hours daily. This is a lessened daily average of sunlight exposure as compared to the June experiment. There is the further probability that the antirachitic intensity of the solar radiation during the September-October experiment was less than during the June experiment, but it should be remembered that these animals had been exposed to sunlight during the entire summer.

During this experimental period quantitative collection of all excreta was made and the feed, milk, urine, and feces analyzed for calcium. The records of the data secured in the metabolism experiment of September are shown in Tables IV, V, and VI.

TABLE IV.

*September Record of Calcium Balance and Milk Production of Cow 2.*

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week
Timothy hay—sunlight period.							
	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
Sept. 14–21.....	420.9	2.5	177.9	601.3	531.0	−70.3	256.2
“ 21–28.....	366.4	2.5	190.2	559.1	531.0	−28.1	258.4
“ 28–Oct. 5.....	340.7	1.1	182.8	524.6	531.0	+6.4	247.1
Oct. 5–12.....	297.2	1.1	165.7	464.0	531.0	+67.0	250.1

TABLE V.

*September Record of Calcium Balance and Milk Production of Cow 3.*

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Timothy hay—sunlight period.							
	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
Sept. 14–21.....	429.2	1.7	118.9	549.8	518.3	−31.5	194.0
“ 21–28.....	404.4	3.1	115.7	523.2	518.3	−4.9	193.1
“ 28–Oct. 5.....	341.2	5.0	114.6	460.8	518.3	+57.5	188.9
Oct. 5–12.....	316.8	1.1	115.1	433.0	518.3	+85.3	182.5

TABLE VI

*September Record of Calcium Balance and Milk Production of Cow 4.*

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Timothy hay—sunlight period.							
	gm.	gm.	gm.	gm.	gm.	gm.	lbs.
Sept. 14–21.....	453.6	1.5	195.8	650.9	543.7	−107.2	302.2
“ 21–28.....	432.7	8.1	228.9	669.7	543.7	−126.0	296.6
“ 28–Oct. 5.....	415.3	9.1	218.2	642.6	543.7	−98.9	287.8
Oct. 5–12.....	379.9	4.4	166.7	551.0	543.7	−7.3	253.3

A study of the records show that Cow 2 (Table IV) was in negative calcium balance for the first 2 weeks of the experiment and in a positive balance for the next 2 weeks. For the total experimental period of 4 weeks the animal was negative some 30 gm. of  $\text{CaO}$ . At this time she was producing about 250 pounds of milk per week in contrast to 400 pounds per week during June when a more distinct negative calcium balance prevailed, even in the sunlight period. It may well be questioned whether there is a direct relation between the ration plus the environment on the one hand and sustained milk flow on the other, and whether the milk flow will recede to a point where the ration plus the environment can sustain the organism in a condition of equilibrium. This equilibrium may apply to energy, nitrogen, phosphorus, calcium, or any of the elements or compounds entering into the constitution of a normal milk. We realize that there are certain constituents of milk that are variable dependent upon the food supply and the suggestion offered above may not apply to such substances. Further, as is well known, a milch cow may sacrifice her own tissues for milk production up to a certain point.

In the case of Cow 3 (Table V) negative lime balances prevailed during the first 2 weeks of the September sunlight experiment, but distinct positive balances were recorded for the last 2 weeks. For the entire period of 4 weeks a positive balance of 106 gm. of calcium oxide was recorded. While this cow was in negative lime balance during the June sunlight experiment when she was giving approximately 300 pounds of milk per week, she was now in equilibrium, but with a production of 190 pounds of milk per week or about 27 pounds daily. This is a comparatively low milk production.

In the case of Cow 4 (Table VI) negative lime balances were recorded through the entire experimental period. This negativity of lime amounted to 333 gm. for the 4 weeks. This cow was fresh in August, 1925, and the most liberal milker in the group used in the September experiment. She was producing about 300 pounds of milk per week when the experiment started and 250 pounds at the end of the metabolism trial. This was a daily production of about 40 pounds of milk.

Consideration of all the data secured in the two metabolism trials indicates that with such rations as were used insufficient lime is assimilated to meet the needs of maintenance and milk

production even in sunlight. Only when milk production falls to 25 to 30 pounds per day was the ration used, *plus sunlight*, able to maintain calcium equilibrium. It should be emphasized again that the ration of corn silage, green grasses, and grains, or corn silage, timothy hay, and grains compares favorably with the average ration fed dairy cows in the northern states during the summer time. The sunlight factor, while of some moment in increasing the utilization of lime in the milking animal, nevertheless was insufficient in its potency to turn negative lime balances to positive ones with the ration used and with cows milking liberally, that is above 35 pounds of milk per day. Under the constant drains of liberal milk production sunlight seemed to be relatively feeble in protecting the animal against constant lime losses. Only when milk production had decreased to much lower levels than in the early periods of lactation was calcium equilibrium established. This apparently feeble action of sunlight as contrasted with the radiations of a quartz mercury lamp merits important consideration for future studies in milk production by dairy cattle.

This investigation raises other important questions: First, is the maintenance of calcium equilibrium—provided all other nutritional requirements are met—necessary for the maintenance of a high and sustained milk flow? While no long metabolism experiments involving high and low lime rations have been made to determine this point, yet actual feeding experiments involving a measure of milk yield have been made by Meigs (6) with low and high lime rations. These experiments answer the question raised in the affirmative. Cows on timothy hay (low lime ration) showed an average drop in milk yield of about 50 per cent at the end of 4 months after freshening. While those receiving alfalfa hay (liberal lime ration) showed a drop of but 25 per cent in the same time. Similar results (7) have been secured by us and by Orr, Chrichton, Chrichton, and Middleton (8). Since the protein differences between timothy and alfalfa hay were met by a provision of more liberal intake of protein on the timothy hay ration, the differences in milk yield are attributable to the differences in lime intake of the two rations. This view is further confirmed by the fact that supplementing the timothy hay ration with lime

gave results in milk yield comparable to the alfalfa ration, at least for a time. It must be remembered, however, that mere provision of a bountiful supply of lime may not insure continued high milk production and calcium equilibrium. It is entirely possible, as has been shown in the case of laying hens (9), that the combination of high lime plus a generous supply of the antirachitic factor is necessary for a continued liberal milk production.

Second, can calcium equilibrium be met by the use of high calcium-containing legumes such as alfalfa or clover *plus sunlight*, particularly with high producing cows? Or has the dairy cow been bred to such a high milk-producing capacity that no natural environment of feed and sunlight can maintain the calcium requirement in the best producers without provision of extra supplies of the antirachitic factor; and instead of long continued milk secretion in the case of liberal milk producers we will generally see, unless extra sources of the antirachitic vitamin are provided, a lowering of milk flow as lactation proceeds, an impairment of the health, and a train of disturbances exhibited by poor calf production or nutritional abortion or sterility—all possibilities under the stress of a long continued rachitic condition. This is another question that awaits an answer.

#### SUMMARY.

1. Cows milking 45 to 60 pounds of milk daily were in negative lime balance on a ration of grains, grain supplements, corn silage, and green grasses when the experiment was conducted out of contact with direct sunlight.

2. On the same ration but with the experiment conducted in direct sunlight in June, negative lime balances still continued, although the degree of negativity was somewhat reduced. Sunlight plus a ration with a lime content comparable to the average ration fed dairy cows in the summer time did not maintain calcium equilibrium in these animals. These cows were milking 45 to 60 pounds of milk daily.

3. On a ration with timothy hay substituted for the green grasses and with a lime content similar to that of the grasses used negative lime balances prevailed in September sunlight with cows milking 40 to 45 pounds of milk daily. With cows milking 25 to 30 pounds

of milk daily—that is relatively poor production—calcium equilibrium was maintained with such a ration plus September sunlight.

4. Apparently summer sunlight in comparison with the radiations of a quartz mercury vapor lamp is feeble in its antirachitic properties when considered in relation to liberally milking animals. For the maintenance of calcium equilibrium in liberally milking cows higher lime intakes than are contained in the ration we used must be provided even in *summer* feeding. It is certainly still a question whether a generous lime intake in the *winter* feeding of milch cows of high production will maintain the desired lime equilibrium. Certainly most records show negative lime balances under indoor feeding conditions even when a lime-rich roughage such as alfalfa hay has been used. Possibly resort must be made, particularly during winter feeding, to an additional supply of the antirachitic factor.

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## FURTHER OBSERVATIONS ON THE FATE OF ACID IN THE BODY.

By CYRUS H. FISKE, ROBERT A. GOODELL, LOUIS E. HATHAWAY, JR., AND EDWARD J. WEST.

(From the Biochemical Laboratory, Harvard Medical School, Boston.)

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Aside from experiments on rabbits, which are in a special class, Kurtz<sup>1</sup> and Gaehtgens<sup>2</sup> were the first to show a definite loss of fixed base in the urine after feeding acid.

In the first of Gaehtgens' two experiments, in which dogs were used as subjects, the diet was extracted meat. The administration of acid (sulfuric) increased the excretion of sodium and potassium about equally; in the afterperiod there was possibly some retention of sodium, but the potassium output did not fall below the normal level. In the second experiment, the dog was fed fresh meat and fat, and here the rise in fixed base was limited to potassium.<sup>3</sup> The analytical figures indicate, however, that both sodium and potassium were retained after the administration of acid had been stopped.

Stehle<sup>4</sup> (evidently unaware of Gaehtgens' publication) gave a dog about 85 cc. of 0.1 N glycine hydrochloride per kilo on each of 3 successive days. Apparently both sodium and potassium were lost (in roughly equal quantities), and the lost base nearly if not quite all regained. But the urine in the 4 day preliminary period was far from uniform in composition, and the magnitude of the effect of acid hence cannot be accurately gauged. The same dog—before recovery from the first experimental period was complete—was fed free hydrochloric acid (approximately 30 to 60 cc. of 0.1 N per kilo) on 11 out of 12 consecutive days. The results in this case were qualitatively the same, though less pronounced. The experiment as a whole does not differ materially from the work of Gaehtgens, except that the acid period was longer.

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<sup>1</sup> Kurtz, J., Inaugural dissertation, Dorpat, 1874; cited by Gaehtgens.<sup>2</sup>

<sup>2</sup> Gaehtgens, C., *Z. physiol. Chem.*, 1880, iv, 36.

<sup>3</sup> We are not now considering calcium and magnesium, since to judge from previous work they are not important factors in the neutralization of administered acid.

<sup>4</sup> Stehle, R. L., *J. Biol. Chem.*, 1917, xxxi, 461.

Stehle and McCarty<sup>5</sup> subsequently published the results of two experiments on human subjects. Among similar experiments on record, they stand virtually alone in the small amount of ammonia produced, and in the correspondingly large output of fixed base. The reason obviously is that they were incomplete. Acid was fed for 3 days in succession, and the collection of urine suspended at that point; that is, there was no afterperiod. From the standpoint of ammonia production, a comparison with the second experiment of Begun, Herrmann, and Münzer<sup>6</sup> is illuminating. There the subject likewise took acid for 3 days, and the extra ammonia that had been excreted by the end of the 3rd day was little more than half the total yield at the conclusion of the afterperiod.

A further partial explanation of Stehle and McCarty's atypical results may be found in the diet which they used. From the pH, the low ammonia excretion, and other characteristics of the urine before the acid was administered, the diet was evidently near the border-line between acid and alkaline (in the sense of Sherman and Gettler<sup>7</sup>). The possibility of some storage of fixed base during the preliminary period must therefore be considered.

Two experiments of a similar nature on normal children (along with several others on a subject with nephritis) have recently been published by Gamble, Blackfan, and Hamilton.<sup>8</sup> They used, not free acid, but "acid producing" salts (calcium chloride and ammonium chloride), fed for a 6 or 8 day period. Under these conditions it is clear that the fixed base loss is small except during the first few days of experimental acidosis, and further that the loss on the 1st day is chiefly sodium. The excess sodium excreted on that day was equivalent to about 25 per cent of the dose of calcium chloride, on the 2nd day slightly less, and from that point on the sodium output fell to normal or below. The potassium both rose and fell more gradually, and in the afterperiod both bases were definitely retained.

Somewhat earlier, in an experiment performed with no attempt to regulate the diet accurately, Haldane, Wigglesworth, and Woodrow<sup>9</sup> had also noted the temporary character of the rise in sodium excretion, but the increase on the 1st day in their case was very much more marked (equivalent to about 85 per cent of the dose of ammonium chloride). The subject was evidently on a very high sodium intake, a circumstance which probably explains the difference in their findings, especially since Gaetgens' dog, on a diet of low sodium content, showed little or no loss of sodium.

A rise in sodium output, when it does occur, might represent base used to neutralize the acid, or merely sodium chloride incidentally washed out in

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<sup>5</sup> Stehle, R. L., and McCarty, A. C., *J. Biol. Chem.*, 1921, *xlvi*, 315.

<sup>6</sup> Begun, A., Herrmann, R., and Münzer, E., *Biochem. Z.*, 1915, *lxxi*, 255.

<sup>7</sup> Sherman, H. C., and Gettler, A. O., *J. Biol. Chem.*, 1912, *xi*, 323.

<sup>8</sup> Gamble, J. L., Blackfan, K. D., and Hamilton, B., *J. Clin. Inv.*, 1924-25, *i*, 359.

<sup>9</sup> Haldane, J. B. S., Wigglesworth, V. B., and Woodrow, C. E., *Proc. Roy. Soc. London, Series B*, 1924, *xvii*, 1.

consequence of the diuresis. In acidosis brought about by feeding hydrochloric acid (or a salt which gives rise to hydrochloric acid in the body), it is evidently impossible to make a choice between these two alternatives, at least on anything like a quantitative basis. Under other circumstances, analysis of the urine might lead to much too low an estimate of the amount of sodium appropriated by the acid. Sodium chloride fed as such is sometimes retained in the tissues for periods of considerable length,<sup>10</sup> and sodium chloride formed within the body (*e.g.* from hydrochloric acid and sodium bicarbonate) might suffer the same fate, especially in a subject on a salt-poor diet.

Considerations of this nature, whether or not they would furnish a complete explanation of the widely divergent findings recorded in the literature from time to time, nevertheless testify to the advantage of administering an acid which, if not a foreign substance altogether, is at least primarily a waste product rather than an important constituent of the tissues.

Occasionally a loss of sodium alone has been reported. This was the case in the experiment of Dunlop,<sup>11</sup> which was not properly controlled. Shohl and Sato,<sup>12</sup> working with infants, likewise came to that result; here the element of rapid growth may be in part responsible, for absence of any loss of potassium whatever is surely not the usual outcome.

The fact that the nature of the diet, which has varied greatly in the work referred to, may not be wholly without influence, makes it all the more desirable to investigate the effect of acid when all food has been withheld.

In a previous paper, Fiske and Sokhey<sup>13</sup> reported on the disposition of comparatively small amounts of acid (12.5 to 30 cc. of 0.1 N per kilo) fed to fasting cats. In most of their experiments about 60 per cent of the acid recovered from the urine was found to be in combination with ammonia, and the same figure is very near the average for them all. The fraction neutralized by fixed base—averaging about 30 per cent—was subject to wider fluctuations, and was sometimes hardly large enough to be detected. Because of these irregularities it was argued that the lost fixed base was probably in every instance taken from a variable reserve supply, which the body is under no particular obligation to retain.

An average fixed base loss equivalent to 30 per cent of the acid fed is not radically different from the *net* result of most earlier

<sup>10</sup> See, *e.g.*, Adolph, E. A., *J. Physiol.*, 1921, lv, 114.

<sup>11</sup> Dunlop, J. C., *J. Physiol.*, 1896, xx, 82.

<sup>12</sup> Shohl, A. T., and Sato, A., *J. Biol. Chem.*, 1923-24, lviii, 235, 257.

<sup>13</sup> Fiske, C. H., and Sokhey, S. S., *J. Biol. Chem.*, 1925, lxxiii, 309.

*investigations.* There is no doubt, however, that much more base than that may be withdrawn at first,<sup>2, 5, 8, 9</sup> and subsequently in part restored by a process of compensatory retention.<sup>1, 4, 8, 9</sup> Nothing of this kind has hitherto been seen except in subjects that were fed, and there the material withdrawn may largely represent an unessential surplus, which is later on replaced by bases from the food.

In the experiments of Fiske and Sokhey<sup>13</sup> on fasting animals no sign of such retention could be found. This point might have been offered as additional evidence that the tissues were not robbed of indispensable fixed base, but at that time it was not known whether retention of base liberated from the tissues in the fasting state is possible at all.

Partly to determine whether compensatory fixed base retention ever can occur in inanition, we have undertaken now to see what happens when the dose of acid is several times as large. The limit is set, not by the effect of acid on the body as a whole, but by vomiting—a stumbling block that has been met before.<sup>14</sup> The tendency to regurgitation appears to be a matter of individual susceptibility; cats that will retain a given dose of acid once will do so on subsequent occasions too. Of this fact, which would have been the means of saving us much time if we had known it, we were not aware until too late to turn it to advantage.

The dose selected was 100 cc. of 0.1 N sulfuric acid per kilo, as far as we know the largest single dose that has been given in similar experiments including determinations of fixed base. The rate of absorption of acid into the circulation under these conditions must for a time be very much greater than in the severest type of diabetic acidosis. This must be kept in mind in comparing the results with previous work.

The most extreme case of ketonuria in a diabetic known not to be receiving alkali appears to have been found by Schwarz.<sup>15</sup> The rate of acetone body excretion in his patient—due allowance being made for the fact that at least half the  $\beta$ -hydroxybutyric acid can be excreted uncombined with base<sup>16</sup>—would be equivalent to about 3.5 cc. of 0.1 N mineral acid per

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<sup>14</sup> Walter, F., *Arch. exp. Path. u. Pharmacol.*, 1877, vii, 148. Eppinger, H., and Tedesko, F., *Biochem. Z.*, 1909, xvi, 207.

<sup>15</sup> Schwarz, L., *Deutsch. Arch. klin. Med.*, 1903, lxxvi, 233.

<sup>16</sup> Henderson, L. J., and Spiro, K., *Biochem. Z.*, 1908, xv, 105.

kilo per hour. In cats which have ingested 100 cc. of 0.1 N sulfuric acid per kilo the rate of excretion of the acid radicle reaches figures at least three times as high. For example, a cat of 3 kilos, excreting sulfate during a preliminary control period at the rate of 4.0 cc. of 0.1 N per hour, was given 100 cc. of 0.3 N sulfuric acid *per os*. The inorganic sulfate excretion for the next 5 hours was as follows: 21.2, 34.8, 35.3, 31.0, and 22.9 cc. of 0.1 N (per hour). Corrected for the control period, and divided by the body weight, the figures for excess sulfate excretion become 5.7, 10.3, 10.4, 9.0, and 6.3 cc. of 0.1 N per kilo per hour.

An estimate of the velocity of absorption of the sulfate radicle under these conditions is also of some interest. Following the intravenous injection of sodium sulfate, we have found that its excretion is approximately logarithmic; about 25 per cent of the injected sulfate remaining in the body at any given moment is excreted in the succeeding hour. Hence the excretion rate attained in the above experiment could hardly have been possible unless the velocity of absorption was in the neighborhood of 40 cc. of 0.1 N per kilo per hour.

Although the acidosis associated with exercise may be much more severe, there is at the present time no evidence that it ever brings about a loss of base (either fixed base or ammonia) at a rate approaching 10 cc. of 0.1 N per kilo per hour. Physical exertion mild enough to be sustained for several hours evidently leads to very little loss of lactic acid.<sup>17</sup> In very vigorous exercise, which can be kept up for only a few minutes at the most, lactic acid excretion may reach higher levels, but the maximum so far observed in any hourly period is only 3 or 4 cc. of 0.1 N per kilo.<sup>18</sup> Within less than 1 hour after exercise of this variety the lactic acid has practically disappeared.

#### EXPERIMENTAL.

The inorganic phosphate determinations were made by the method recently described by Fiske and Subbarow;<sup>19</sup> aside from this the analytical methods were identical with those used before.<sup>13</sup> In other experimental details as well there was no departure from the previous procedures, except in one respect necessitated by the larger dose of acid. That is, the sulfuric acid was administered (by stomach tube as usual) in a volume of 100 instead of 25 cc.; on the following day we then gave only 25 (rather than 100) cc. of water, with the obvious purpose of preventing an excessive rise in urinary volume.

<sup>17</sup> Ryffel, J. H., *J. Physiol.*, 1909-10, xxxix, p. xxix. Campbell, J. A., and Webster, T. A., *Biochem. J.*, 1922, xvi, 106.

<sup>18</sup> Liljestrand, S. H., and Wilson, D. W., *J. Biol. Chem.*, 1925, lxxv, 773.

<sup>19</sup> Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, lxxvi, 375.

Owing to the trouble that we had with vomiting, only two satisfactory experiments could be completed in the time during which we were able to collaborate. Although these two experiments were not just alike, both gave substantially the same results on the chief points that we intended to investigate when we began. The second cat (Experiment 2) received two separate doses of acid, alike in size; one of them before the collection of urine was begun, the other 1 week later. The preliminary period of this second experiment hence corresponds with the afterperiod of the first (Experiment 1).

The analytical data are presented in Table I, while Table II contains the ratios of ammonia and residual fixed base,<sup>20</sup> respectively, to total nitrogen, as well as the percentage of the acid neutralized by each from day to day. Inasmuch as the sulfuric acid was wholly recovered from the urine, within a reasonable margin of error, we have this time based our calculations on the total dose.

### Results.

*Ammonia.*—The immediate response of the ammonia mechanism is clearly not proportional to the dose of acid. With 25 cc. of 0.1 N acid per kilo, about 60 per cent is neutralized by this device<sup>13</sup> (all within the limits of one 24 hour period); a fourfold increase in the dose only doubles the amount of extra ammonia produced on the 1st day. In both experiments alike, although all or nearly all the extra sulfate was excreted within 24 hours of the acid feeding, only about 30 per cent of it turned out to be in combination with ammonia. The ammonia output however remained high for at least 3 additional days, and the total quantity formed eventually approached the 60 per cent observed before. Our experiments were continued only so long as the ammonia coefficient remained well above the normal level. On the last day in each case it was still perceptibly higher than it had been just before the acid period, and the yield of ammonia might possibly have been larger if more time had been allowed. On the other hand, the gradual excretion of traces of excess ammonia is difficult to detect with any

<sup>20</sup> Residual fixed base = total fixed base minus chloride. See the paper of Fiske and Sokhey,<sup>13</sup> where a discussion of the method of calculation also will be found.

certainly, for the ammonia coefficient shows some variation normally.

*Fixed Base.*—In general, the fixed base excretion follows the course that might have been inferred from the behavior of ammonia. On the 1st day, a somewhat larger fraction of the sulfuric acid was excreted in combination with fixed base than was the case with smaller doses. Corresponding with the continued high ammonia production, a considerable part of this lost substance (about one-third) was later on retained. The point of perhaps the greatest general interest is that a fasting animal is capable of appropriating fixed base, set free by the autolysis of its own tissues, to replace lost material. In other words, base retention evidently can occur without a food supply to draw upon; hence the reason it could not be detected in the earlier experiments with less acid<sup>13</sup> must be that the need for it did not exist.

Circumstances prevented the immediate completion of the fixed base determinations, and we had only the ammonia to go by in deciding when the experiments should be stopped. As it was, the ratio of residual fixed base to total nitrogen did not return to normal, so the amount of fixed base retained is unquestionably greater than our figures indicate. The residual fixed base is in fact still low even after the expiration of a week. The 7th day of fasting in Experiment 2 was also the 7th day after the first dose of acid, and at that time the ratio was only 22, as compared with the normal of approximately 35.<sup>13</sup> For this reason also, our estimate of the net fixed base loss after the second dose of acid is undoubtedly too high, and the figures have on that account been bracketed (Table II). They were calculated on the supposition that the base-nitrogen ratio would have remained at 22 if acid had not been given a second time. If, instead, the normal ratio (35) is taken as the basis for the calculations, the fraction neutralized by fixed base would come to 29 per cent instead of 55. The true result must lie between these two extremes, and so is not unlike the outcome of the first experiment.

It is worthy of note that the second dose of acid in Experiment 2 caused, temporarily, the retention of virtually all the residual fixed base that was set free. The total fixed base content of the urine on the 2nd day of the acid period was hardly more than the equivalent of the chloride; the residual fixed base, in other words, fell practically to zero.



Increased fixed base excretion after feeding acid is not enough, alone, to show that the demand for ammonia actually exceeds the possible supply.<sup>21</sup> More convincing is the fact, brought out by this investigation,<sup>22</sup> that less ammonia in proportion is pro-

TABLE I.  
*Sulfuric Acid per Os (100 Cc. 0.1 N per Kilo).*

Day of fast.	Urine.							Remarks.
	Volume.	Total N.	Inorganic P.	Inorganic sulfate.	Ammonia.	Residual fixed base.*	Cl	
Experiment 1. Fasting cat. 2.35 kilos.								
	cc.	gm.	mg.	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	
7	101	0.97	67	24	37	36	13.9	
8	98	1.04	86	29	42	36	10.7	
9	100	1.10	83	29	42	36	12.8	
10	172	1.09	54	255	113	172	10.8	100 cc. 0.235 N H <sub>2</sub> SO <sub>4</sub> (equivalent to 235 cc. 0.1 N).
11	93	1.09	74	48	66	30	12.0	
12	111	1.04	64	27	57	17	12.5	
13	104	1.00	60	28	49	16	9.8	
14	87	0.89	59	22	38	20	6.3	
Experiment 2. Fasting cat. 2.24 kilos.								
4	115	1.44	69	29	60	23	18.9	
5	72	1.06	60	20	45	22	3.1	
6	66	1.01	56	23	41	23	2.3	
7	98	1.07	53	23	39	24	1.4	
8	160	1.43	114	239	125	196	4.6	100 cc. 0.224 N H <sub>2</sub> SO <sub>4</sub> (equivalent to 224 cc. 0.1 N).†
9	53	0.96	43	22	56	1	14.3	
10	71	0.96	57	23	47	7	9.7	
11	94	0.94	50	24	40	12	4.0	

\* Total fixed base minus chloride.

† The cat had previously been given the equivalent of 100 cc. of 0.1 N sulfuric acid per kilo on the day the fasting was begun.

<sup>21</sup> Cf. the intravenous injection experiments of Fiske and Sokhey.<sup>13</sup>

<sup>22</sup> A comparison of the effects of widely different quantities of acid, when the conditions are otherwise the same, has not previously been attempted to our knowledge.

duced at first when the dose of acid is increased. But we are not prepared to say that even now we have subjected the ammonia mechanism to a task beyond its powers. On the other hand, whether fixed base was lost because ammonia could not be manufactured rapidly enough, or whether the reason is—as we

TABLE II.

Day of fast.	Base-nitrogen ratios.		Per cent of acid neutralized by:			N:P
	Ammonia per gm. N.	Residual fixed base per gm. N.	Ammonia.	Residual fixed base.	Sum.	
Experiment 1.						
	cc. 0.1 N	cc. 0.1 N				
7	38	37				14.5
8	41	35				12.1
9	38	33				13.3
10*	104	158	31	58	89	20.3
11	60	28	10	-2	8	14.7
12	55	17	8	-7	1	16.1
13	49	16	5	-7	-2	16.7
14	43	23	2	-4	-2	15.0
Sum .....			56	38	94	
Experiment 2.						
4	42	16				20.9
5	42	21				17.6
6	41	22				17.9
7	37	22				20.0
8*	87	137	32	(74)	(106)	13.8
9	58	1	9	(-9)	(0)	22.5
10	49	7	5	(-6)	(-1)	16.8
11	43	13	3	(-4)	(-1)	18.8
Sum.....			49	(55)	(104)	

\* Day of acid administration.

suspect—a different one, there can be no doubt that in this case something more than an unessential surplus has been tapped. The subsequent fixed base retention—since the animals were fasting—admits no other explanation.

*Phosphate.*—The persistently low output of fixed base after the

ammonia has returned to normal must have its counterpart among the acid excretory products. The organic acid excretion, though showing some tendency to fall progressively in fasting, is not perceptibly diminished by feeding acid.<sup>23</sup> The phosphate on the contrary does fall, although acidosis is commonly supposed to have the opposite effect. The rise in both total nitrogen and phosphate in Experiment 2 obviously means only that the acid has increased the rate of tissue breakdown, as it has been known to do before.<sup>24</sup> Evidence for a specific effect on phosphate metabolism, in either one direction or the other, cannot be adduced from the phosphate excretion by itself, but only from the N:P ratio. Under the influence of acid the values of this ratio are quite unmistakably high (Table II). For comparison we give below the corresponding ratios for 8 control experiments on fasting cats, each figure representing a 4 day period beginning with the 3rd or 4th day of inanition: 12.7, 13.0, 13.2, 13.3, 13.5, 13.7, 14.2, 14.9. Further, 78 separate 24 hour urines, collected between the 3rd and 13th days of fasting, showed a mean N:P ratio of 13.8 (standard deviation 0.85). In many of them both total and inorganic phosphorus were determined; the difference between the two is hardly outside the limit of error of the colorimetric method. Normally the ratio rarely is as high as 16, while figures in the vicinity of 20—several of which will be found in Table II—never have been seen in our controls.

The retention of phosphate after feeding acid may be a peculiarity of fasting subjects. In these experiments, moreover, it is evidently associated in some way with the retention of fixed base, and possibly will not occur except in conjunction with the latter process.

*Sodium and Potassium.*—Investigation of the behavior of these bases individually has been hampered by lack of the necessary analytical technique. The determination of sodium when its amount is very small is beyond the scope of ordinary methods, and we have been obliged to calculate it from the difference

<sup>23</sup> The organic acid content of urine may be calculated from the data given, combined with the titratable acidity determined on a sample freed of CO<sub>2</sub>.

<sup>24</sup> McCollum, E. V., and Hoagland, D. R., *J. Biol. Chem.*, 1913-14, xvi, 299.

between the total base and the sum of the potassium, calcium, and magnesium. Under the circumstances the potassium content must be known more accurately than the cobalti-nitrite method is capable of determining it. A more precise potassium method has been developed in this laboratory, but was not ready for use until most of the work reported here had been completed.<sup>25</sup> A preliminary note on some of the results which we have so far gathered may however be of interest, inasmuch as this aspect of the subject has not been touched upon before with fasting animals.

The rise in fixed base on the 1st day after feeding 100 cc. of 0.1 N sulfuric acid per kilo is about equally divided between sodium and potassium. This serves to show that a sufficiently large dose of acid will remove sodium even if no food containing it is eaten—a result that is a bit surprising, considering the tenacity with which sodium is retained in fasting.<sup>26</sup> On the other hand it follows that a large part of the base has come from sources other than the mobile body fluids—even the corpuscles of the cat contain very little potassium.<sup>27</sup>

In the afterperiod, sodium retention is most in evidence at first; the sodium content of the urine may for a time be vanishingly small.<sup>28</sup> The retention of potassium, which from present indications is slower in appearing, persists however for a longer time—the base which is still being retained a week after the acid has been fed is probably potassium alone. Although sodium may disappear entirely from the urine, the amount of potassium that can be retained is evidently limited. The urine of normal fasting cats contains in the neighborhood of 30 cc. of 0.1 N potassium per gm. of total nitrogen. During the retention period the level may fall to about 30 per cent of the normal output, but lower than that it does not seem to go, even if acid is given a second time.

<sup>25</sup> We are indebted to Dr. Georges Litarczek for several potassium analyses.

<sup>26</sup> Hendrix, B. M., and Sanders, J. P., *J. Biol. Chem.*, 1923-24, lviii, 503.

<sup>27</sup> Abderhalden, E., *Z. physiol. Chem.*, 1898, xxv, 65.

<sup>28</sup> This is true even if the chloride output rises, as it sometimes does early in the afterperiod (*cf.* Experiment 2, Table I).

## SUMMARY.

In fasting cats, within the first 24 hours after feeding a comparatively large amount of acid (100 cc. of 0.1 N per kilo), there is relatively more loss of fixed base and less ammonia production than when the dose is smaller. Both sodium and potassium are lost, and later they are both retained, while the ammonia excretion continues to be high for several days—indicating that enough fixed base had been withdrawn to alter the composition of the tissues.

Coincident with the retention of fixed base, phosphate also is retained, as evidenced by a rise in the N:P ratio in the urine.

## CLINICAL CALORIMETRY.

### **XL. THE EFFECT OF THE ABSENCE OF SWEAT GLANDS ON THE ELIMINATION OF WATER FROM THE SKIN AND LUNGS.**

BY HENRY B. RICHARDSON.

WITH THE TECHNICAL ASSISTANCE OF G. F. SODERSTROM.

*(From the Russell Sage Institute of Pathology in Affiliation with the Second Medical (Cornell) Division of Bellevue Hospital, New York.)*

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#### INTRODUCTION.

The rare condition known as congenital ectodermal defect, in which the sweat glands are totally absent, gives opportunity to study the excretion of water by these glands as distinguished from the epithelium of the skin. The patient to be described, Chas. R., was studied both at rest and during exercise, by means of the respiration calorimeter of the Russell Sage Institute of Pathology. The effect of external heat was also noted, as well as the partition of water elimination between skin and lungs. Opportunity to observe this patient was given through the courtesy of Dr. George M. MacKee.

#### LITERATURE.

Du Bois (1) has discussed the vaporization of water and a review of the literature on this subject may be found in Paper XXV of this series (2). The observations on congenital ectodermal defect have been summarized in the paper by MacKee and Andrews (3), which includes a summary of our calorimeter findings. Additional reference need be made here only to the work of Loewy and Wechselmann (4), who made an exhaustive study of three patients, with observations on the excretion of water through the skin. They found that these individuals, though devoid of sweat glands, were able to give off a normal quantity of water vapor from the skin under ordinary resting conditions, but not when either the heat production or the external temperature was raised. Corresponding to this inability to meet changed conditions, an undue elevation of the body temperature was observed. Evidence of a compensatory mechanism was found in the increased volume of respiration observed under the influence of direct heat.

*Case History.*

The detailed history and physical findings will be found in the paper of MacKee and Andrews (3). The patient, Chas. R., schoolboy, was born on Sept. 5, 1908, and was admitted to the metabolism ward on Dec. 28, 1922. He was therefore 14 years of age when under observation. His weight was 45.5 kilos and his height 160.9 cm. He complained that he could not play in the summer time because if he did so he became hot and had to stop, though he had no difficulty so long as he remained still. As might be expected, he was not affected by damp or muggy weather, but only by the increased temperature of the air. He made a practice of wetting his shirt on hot days and was thereby enabled to exercise with less discomfort. The physical examination showed the findings and appearance characteristic of congenital ectodermal defect, and three sections of the skin, taken by MacKee and Andrews (3), one from the extensor surface of the left forearm and two from the left cheek, showed no trace of sweat glands. In spite of this the skin felt normal as to moisture.

*Preliminary Remarks.*

The total heat produced by an individual, as measured in the calorimeter by the direct method (5), comprises three parts. The first is given off by radiation and conduction, and is removed from the calorimeter by means of a radiator through which runs a current of cool water. A small correction for change in temperature of the walls of the calorimeter is required. The second portion is lost by evaporation of water from the skin and lungs, and for every gm. of water vaporized at 20°C. 0.586 calories are removed (6). For slightly higher temperatures the figure is 0.584.<sup>1</sup> The sum of the heat of radiation and conduction, plus that of vaporization, constitutes the heat eliminated. When the total heat production is to be ascertained a third factor must be introduced, the correction for the change in body temperature. This change, multiplied by the weight of the body and by its specific heat, gives the calories stored in or lost from the body. The specific heat is assumed to be 0.83.

*Method.*

By means of the calorimeter, the entire metabolism could be studied, including the respiratory exchange, the direct heat, and

<sup>1</sup> When a subject is exposed to hot air the cooling effect produced by evaporating a gm. of water is less than 0.584 but the difference is negligible for our purposes.

the elimination of water from the skin and lungs. The method has the further advantage that the whole body could be included, instead of only one leg, as in the work of Loewy and Wechselsmann (4). The usual technique of the calorimeter was employed (5) and the accuracy can be judged by the alcohol checks of the year, as published in Papers XXXIII (7) and XXXIV (8) of this series. The latter shows an average error in the water determination in 3 hour periods of plus 5.6 per cent, the variations being from plus 2.2 per cent to plus 8.6 per cent. The error in the heat measured varied from  $-2.0$  per cent to plus 5.2 per cent and averaged plus 0.3 per cent. Another check on the accuracy of the apparatus is the comparison of the heat measured directly with that calculated from the respiratory exchange. Using the latter as a standard, we have found, as may be calculated from Table I, that with Chas. R. at rest the direct heat was 6.6 per cent too low, and in the second observation for the effect of exercise, 3.4 per cent too low. With the control, E. F. D. B., the direct heat was 2.3 per cent less than the indirect. It will be seen from Columns 11 and 9 of Table I that the heat, directly measured, was consistently lower than that calculated from the respiratory exchange, and nearly parallel to it.

Both subjects were studied at rest and during exercise, the latter consisting of voluntary movements in imitation of shivering. The temperature of the calorimeter was kept between 24 and 25°C. The latent heat of water vaporized by the subject was considered to be 0.584 calories per gm. In one experiment the vapor from the lungs was measured by passing the expired air by means of a gas mask through a container one-third full of pumice stone saturated with concentrated sulfuric acid. The vessel employed was the ordinary water absorber used for the calorimeter. The increase in weight of the absorber gave the quantity of water vapor expired.

For the study of the effect of external heat an apparatus designed by Mr. O. Newhouse was used, for a description of which the paper of Benedict, Benedict, and Du Bois (10) may be consulted. By means of this device a current of hot dry air could be delivered at the rate of about 500 liters a minute, to a bag in which the patient lay. This bag was made of cloth treated with oil, and was tied around the patient's neck, with no attempt



TABLE I.  
Calorimeter Data.

Date. Weight. Surface area.	Period.	End of period, time.	Carbon dioxide.	Oxygen.	R.Q.	Water.	Urine nitrogen per hr.	Indirect calorimetry.	Heat eliminated.	Direct calorimetry.	Rectal temperature.	Average pulse.	Work added.*	Non-protein R.Q.	Remarks.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	
R															
Jan. 2, 1923 45.5 kg. 1.46 sq. m.	Prelimi- nary. I	12.12									36.08				Basal. Standard breakfast at 6.15 to 6.30 a.m.
		1 12	23.0	21.0	0	80	29.9	69	262	2	63.0	38.11	78	0.80	Fidgeting constantly, except during residuals.
	II	2 12	22 9	20 9	0	80	31.4	68	966	8	64	2 38	05	0.80	Still restless.
	III	3.12	22 7	21.7	0	76	32.1	71.1	69	5	68.4	38	03	0.75	Somewhat quieter.
Average .....			22 8	21.2		31	1	69	866	2	65	2		0.78	
Jan. 4, 1923 45.7 kg. 1.46 sq. m.	Prelimi- nary. I	12.24									38.15	71			Basal moderately restless.
		1.24	22 7	18.7	0	88	30.9	63	565	2	58	0 37	97	0.90	"Shivered" 1.26 to 1.46.
	II	1.59	28 8	25.2	0	83	25.1	84	959	6	85.1	38	65	0.83	Rest. Moderately restless.
	III	2.29	11.5	11	1	0.75	18.0	36	539	1	33	6 38	51	0.74	Very restless.
Average .....			22 8	20	9	0	80	69	379	4	66.9	38.19	27	0.79	
Average .....							0.376								



at an air-tight joint. Part of the air escaped here but most of it through an opening provided for the purpose. The quantity of water eliminated from the skin and lungs combined was determined by weighing the patient before and after on a scale which was sensitive to 10 gm. The change in weight due to the gaseous exchange was neglected, since it came within the error of the scale. Rough though this method may seem, it sufficed to show a marked difference between patient and control.

### *Results.*

The calorimeter data are shown in Table I. The observation on the patient Chas. R. at rest on January 2, 1923, gave the same results as with the control E. F. D. B. during the 1st hour of the observation on May 10, 1922. The former eliminated from skin and lungs in 3 hours an average of 31.1 gm. of water vapor, a quantity which in evaporating would cool the body 18.2 calories, or 28 per cent of the total of 65.2 calories as measured by the direct method. In the control observation 17.3 calories were given off as water vapor out of a total of 66, or 26 per cent. The average for normal individuals is 24 per cent (2).

In the second observation dated January 4, 1923, the patient rested an hour in the calorimeter, and then in the second period, which lasted only 35 minutes, he performed exercise for 20½ minutes. This consisted of movements in imitation of shivering. There followed two resting periods of ½ and 1 hour respectively. For comparison we used the normal data obtained from a previous observation of E. F. D. B., who performed the same type of exercise for a similar period. This subject had a height of 178.5 cm., a weight of 78.0 kilos, and a surface of 1.97 sq. meters, according to the formula of Du Bois and Du Bois (11), or considerably more than the 1.46 sq. meter surface of the patient. In order, therefore, to make the figures comparable, they have been divided through by the respective surface areas and expressed in quantities per hour. These may be found in Table II and Fig. 1. The work performed was similar, being 100 calories for the patient as compared to a basal of 39.7, and 88.6 for the control, as compared to a basal of 33.7.

Much the most striking feature of the exercise is the similarity of the curves of the two individuals. Certain differences, however,

can be detected. In the absence of sweat glands, as shown on the left of the graph, the elimination of water remained at much the same level throughout, whereas with the normal control it rose during exercise from 8.8 to 19.5 calories. There is a difference of 6.5 calories per hour in favor of this individual in the 125 minutes beginning with the exercise period. This may be expressed in another way by means of the percentage of total

TABLE II.

*Effect of Exercise in Absence of Sweat Glands in Terms of Calories per Sq. Meter per Hour.*

Name. Date.	Period.	Radiation and conduction.	Heat of vaporiza- tion	Heat eliminated. ((3) + (4))	Rectal tempera- ture.	Body change correction.	Total heat (direct).	Remarks.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Chas. R. Jan. 3, 1923	Prelimi- nary.				°C. 38 15			Congenital ab- sence of sweat glands.
	I	32.3	12 3	44 6	37.97	-5.0	39.7	Rest.
	II	52 7	17.3	70 0	38.65	+29 9	100.0	Exercise.
	III	39 2	14.4	53 6	38 51	-7.6	46 0	Rest
	IV	40 5	13 9	54.4	38.19	-8 6	45.8	"
E. F. D. B. May 10, 1922	Prelimi- nary.				36.69			Normal con- trol.
	I	27.7	8.8	36 5	36 61	-2.8	33.7	Rest.
	II	46.3	19 5	65.8	37.02	+22 8	88 6	Exercise.
	III	33.5	18 7	52.2	36.99	-2.3	49.9	Rest.
	IV	31.6	13 9	45.5	36.81	-6.1	39.4	"

All of the data have been converted into calories and divided by the body surface to give the results in calories per sq. meter per hour.

heat given off as water vapor. This was 22 per cent during the exercise, and 37 in the period following, the figures for the patient being 17 and 30 respectively. Except for this faulty elimination of water vapor, no difference between the two individuals was noted. The curves showing the heat lost by radiation and conduction are practically identical, though at different levels. No evidence was found that the patient could compensate, by means

of increased peripheral circulation, for the defective elimination of water vapor. As a result of the latter the heat eliminated was somewhat less in proportion to the total than was the case

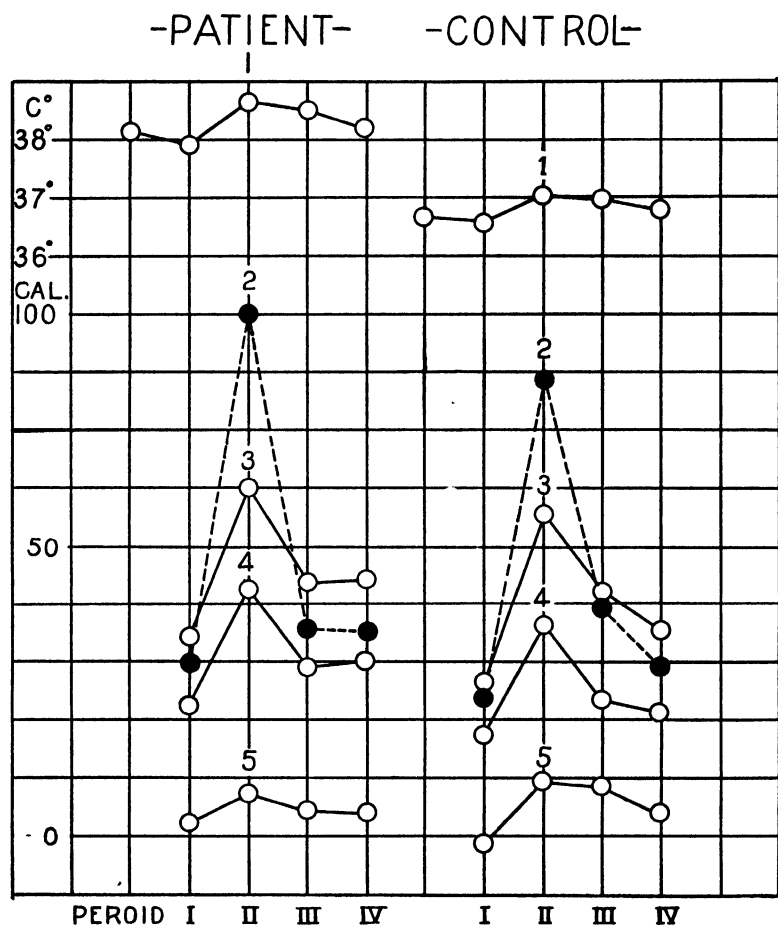


FIG. 1. All the data (except the body temperature) have been converted into calories and divided by the respective body surfaces to give results in calories per sq. meter per hour. Curve 1, body temperature by rectum. Curve 2, total heat production. Curve 3, heat eliminated. Curve 4, heat lost by radiation and conduction. Curve 5, water vaporized.

The patient without sweat glands is shown on the left and the normal individual on the right.

with the control, and the body temperature rose 0.68 degrees, as against 0.41. This fact may be expressed in another way by saying that the storage of heat was greater, 30 instead of 23 calories.

It is evident, therefore, that the patient was able to eliminate enough water through the skin and lungs to maintain body temperature within safe limits, even though his metabolism increased 250 per cent. The results demonstrate how efficiently the normal needs of the organism were cared for without aid from the sweat glands. The only difference noted was a moderate deficiency in the elimination of water vapor, accompanied by a greater increase of body temperature than in the normal individual.

The above results deal entirely with the water vapor eliminated by skin and lungs combined. It was of interest to find out the quantity given off by the skin alone, and this was done by measuring the water given off in the breath by the patient when at rest. The protocol and the calculations follow.

January 8, 1923, Chas. R. Water vaporized from lungs. Gas mask attached to rubber tubing and thereby to sulfuric acid bottle one-third full of pumice stone saturated with sulfuric acid.

*Period I.*

	<i>gm.</i>	
Weight of bottle at end .....	1437.39	3.38 p.m.
" " " " start .....	1435.77	3.28 "
Gain .....	1.62	10 min.

*Period II.* Nose clip applied.

Weight of bottle at end .....	1439.64	3.55 p.m.
" " " " start .....	1437.39	3.45 "
Gain .....	2.25	10 min.

Relative humidity, 50 per cent. Temperature, 25°C.

*Period III.*

Weight of bottle at end .....	1441.57	4.12 p.m.
" " " " start .....	1439.64	4.02 "
	1.93	10 min.

*Period IV.* Mouthpiece in.

Weight of bottle at end .....	1443.74	4.28 p.m.
" " " " start .....	1441.57	4.18 "
	2.17	10 min.

7.97 in 40 min. = 12.0 per hr.

Thus 7.97 gm. of water vapor were given off in 40 minutes, or 12.0 gm. per hour. In a subsequent test this apparatus was found to allow 0.34 gm. to pass through per hour, therefore the total output was 12.3 gm. A deduction must be made for the intake of vapor, the quantity of which may be calculated from the relative humidity (50 per cent) and the temperature of the inspired air (25°C.), provided the minute volume of respiration is known. The latter may be estimated from the output of carbon dioxide (22.8 gm. per hour) and the proportion of this gas normally present in the expired air under basal conditions, *i.e.* not more than 5 per cent, according to the figures published by McCann and Hannon (12). On this basis the maximum net output of water from the respiratory tract of our patient was 9.3 gm. Reference to Table I, Column 7, shows an average elimination of 31.1 gm. of water vapor from all sources, and of this not more than 30 per cent came from the respiratory passages, and the rest from the external surface of the body. It is evident that even in the absence of sweat glands a large amount of water is eliminated from the skin.

The effect of external heat was studied by means of the hot air bag above described. The output of water vapor was measured by the loss of body weight. The temperature of the body was taken by mouth. The protocols follow.

*Protocol I.*

Chas. R. Jan. 3, 1923. Congenital absence of sweat glands.

10.00 a.m. Voided urine.

10.03 " Body weight, 46.41 kilos.

10.08 " Hot air connected with bag.

10.12 " Mouth temperature, 37.5°C.

10.26 " Mouth temperature, 37.6°C. Temperature at outlet of bag, 48.0°C.

10.30 " Temperature at outlet of bag, 48.8°C.

10.35 " Mouth temperature, 38.0°C. Pulse, 88. Temperature at outlet of bag, 48.8°C.

10.42 " Mouth temperature, 38.4°C. Pulse, 102. Temperature at outlet of bag, 49.2°C. Says that he feels hot now.

10.44 " Body weight, 46.39 kilos.

	<i>kg.</i>
Weight at start.....	46.41
" " end .....	46.39

Loss, .....0.02 kg. = 33 gm. per hr.  $\pm 16$ .

After removal from the bag the body temperature rose to 39°C. at 10.50 a.m. and declined gradually, reaching 37.0°C. only after about 4 hours, *i.e.* 3 p.m.

*Protocol II.*

H. B. R. Jan. 3, 1923. Normal individual. Height, 180 cm. Surface, 1.90 sq. m. Age 34.

11.34 a.m. Voided.  
 11.35 " Weight, 70.67 kilos.  
 11.39 " Mouth temperature, 37.0°C.  
 11.40 " Ventilation of bag with hot air started.  
 11.43 " Pulse, 62.  
 11.45 " Temperature at outlet of bag, 43.3°C.  
 11.50 " Temperature at outlet of bag, 45.3°C. to 44.2°C.  
 11.55 " Mouth temperature, 37.0°C.  
 12 05 p.m. Mouth temperature, 36.8°C. Temperature at outlet of bag, 50.5°C. to 48.3°C.

*kg.*

Weight after voiding. 70.48  
 Urine voided. . . . . 0.05

Weight at end. . . . . 70.53  
 " " start. . . . . 70.67  
 " " end. . . . . 70.53

Loss, . . . . . 0.14 kg. in 25 min. = 336 gm. per hr.  $\pm 16$  gm.

The elimination of water by the normal subject was near the average observed by Benedict, Benedict, and Du Bois (10). It may be of interest to calculate how much of the evaporation of water represented elimination of heat by this individual, and how much went to cool the air in the bag. The subject, male, age 34, had a surface of 1.90 sq. meters, an estimated metabolism of 39.5 calories per sq. meter hour, or a total of 75.0 calories to which need be added not more than 10 per cent (10) for the effect of the heat, or 82 calories in all. Since only the head of the subject was exposed to a lower temperature than that of the body, the bulk of the heat was eliminated by the evaporation of water. Even if all of it were so eliminated, only 82 calories would be accounted for out of a total of 197 calories estimated from the loss of water vapor. The remaining 115 calories served to cool the surrounding medium. It is obvious, therefore, that the elimination of water vapor cannot be used in calculating the total metabolism when the surrounding air exceeds the temperature of the body.



These experiments bring out the functional defect even more clearly. The normal individual, H. B. R., was able to tolerate air at a temperature of  $49^{\circ}$  as measured at the outlet of the bag, and this with an actual decrease in the mouth temperature from  $37.0$  to  $36.8^{\circ}\text{C}$ . In so doing he lost in 25 minutes 140 gm. of water from the skin and lungs, or 336 gm. per hour, equivalent to 196 calories. Chas. R., exposed to similar temperature, sustained a rise in mouth temperature from  $37.5$  to  $38.4^{\circ}\text{C}$ . in 34 minutes, at the end of which period he had lost only 20 gm. in weight. It will be seen from Table I, Column 7, that in the calorimeter at rest he gave off 31.1 gm. of water in an hour, thus showing that comparable quantities were eliminated under the two conditions. The 33 gm. per hour eliminated in the bag corresponded to only 19 calories as compared to 196 in the normal.

In another experiment, the patient entered the bag partially dressed and was given a sprinkler with which to wet his underclothing. Sprinkling was not begun for 30 minutes after the beginning of exposure to heat, during which time his temperature rose from  $36.9$  to  $37.3^{\circ}\text{C}$ . With the sprinkling the rise continued somewhat, but reached a maximum of  $37.8^{\circ}\text{C}$ . in another 18 minutes, maintained this level for 21 minutes, and slowly declined to  $37.6$  at the termination of the experiment 27 minutes later. Thus it was demonstrated that the sprinkler functioned as an artificial sweat gland, and though clumsy compared to the normal apparatus, was quite capable of arresting the rise in temperature.

#### DISCUSSION.

The elimination of water vapor by an individual without sweat glands was found to be normal at rest. Of the total heat produced, 28 per cent was eliminated in the form of water vapor, or very close to the normal average of 24 per cent found by Soderstrom and Du Bois (2). Less than 30 per cent of the vapor came from the lungs, and the remainder from the skin. The results confirm the observation of Loewy and Wechselsmann (4) that vapor can be given off from the normal surface of the body without the aid of the sweat glands. The process is probably one of physical diffusion, although active excretion of water by the epithelium of the skin is conceivable. Whatever the

mechanism, sufficient heat was given off in this manner, in combination with other channels, to keep the body temperature within safe limits, in spite of exercise sufficient to raise the metabolism 250 per cent. Here again approximately normal conditions were maintained independent of the sweat glands.

The effect of exercise, though slight, could nevertheless be demonstrated. Considerably less heat than normal was given off in the form of water vapor, and no compensatory increase in radiation and conduction could be detected. As a result the body temperature rose somewhat higher than in a normal individual. That the patient tolerated the exercise as well as he did was due to the relatively low temperature of the air in the calorimeter (24°C.) which permitted the removal of a large amount of heat by radiation and conduction. If the air had been warmer than the body, heat would have radiated inward instead of outward. A rise of body temperature under these conditions has been noted by Linser and Schmid (13) and by Loewy and Wechselsmann (4) who attributed their results to inadequate vaporization of water.

That this explanation is correct was shown by the quantity of water vapor eliminated by our patient in hot air. The amount lost from skin and lungs remained essentially the same as at 24°C. and the temperature of the body rose 0.9°C. in 30 minutes.

That the relation between these two findings was causal was shown by the fact that the rise of temperature could be arrested by sprinkling water on the skin. The normal individual sustained no rise of temperature, because of copious elimination of water vapor. The cooling effect of this amounted to 196 calories per hour, part serving to remove heat from the body, and the rest to cool the surrounding air.

The inference is that in normal individuals under ordinary resting conditions the liberation of water vapor likewise takes place without the medium of the sweat glands. This conclusion was reached by von Willebrand (14) and confirmed by Loewy and Wechselsmann (4) who found with normal individuals that if either the heat production or the surrounding temperature was raised, there occurred at a certain point a sharp rise in the elimination of water, followed shortly by an outbreak of visible sweat. This may be considered evidence of the onset of activity

of the sweat glands. In agreement with the foregoing, we may conclude that the sweat glands constitute an emergency apparatus which is called upon only under exceptional conditions.

#### SUMMARY AND CONCLUSIONS.

1. An individual in whom the absence of sweat glands had been demonstrated by microscopic examination was studied with reference to the elimination of heat and water vapor.

2. At rest a normal quantity of water vapor was liberated. Of this less than 30 per cent came from the lungs and the remainder from the skin. Vaporization of water from the skin independent of the sweat glands was thereby demonstrated.

3. The quantity of water so eliminated was sufficient in combination with heat lost in other ways to keep the body temperature within safe limits even with exercise which increased the metabolism for a period of 35 minutes to two and a half times the resting value.

4. During this exercise the elimination of water did not increase as in a normal individual and the body temperature rose somewhat higher.

5. With exposure to external heat there was no increase in quantity of water vaporized, and the body temperature rose sharply.

6. The study of this patient confirms the theory that the sweat glands constitute an emergency apparatus which is called upon only under exceptional conditions.

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# THE ANTIRACHITIC VALUE OF IRRADIATED CHOLESTEROL AND PHYTOSTEROL.

## V. CHEMICAL AND BIOLOGICAL CHANGES.

By ALFRED F. HESS, MILDRED WEINSTOCK, AND  
ELIZABETH SHERMAN.

*(From the Department of Pathology, College of Physicians and Surgeons,  
Columbia University, New York.)*

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Further experiments have been undertaken to elucidate the nature of the change which is brought about in cholesterol as the result of subjecting it to ultra-violet radiations. In the preceding communication of this series (1) it was reported that cholesterol which has been activated loses its antirachitic power after repeated crystallizations from alcohol. This result had not been expected, in fact the test was undertaken with the purpose of concentrating and increasing the biologic potency of the cholesterol. In view of this experience a test was carried out with cholesterol which had been purified to a greater degree than any preparation which had been employed thus far in these studies. To this end cholesterol crystals were saponified with alcoholic potash and the sterol extracted with ether. The residue of this extract was recrystallized fifteen times from alcohol, and had the following constants: melting point 149 to 150°C.;  $[\alpha]_D^{21} = -38.40^\circ$  in chloroform solution. This cholesterol was irradiated with the mercury vapor lamp for a half hour with the burner at a distance of 1 foot. When 0.25 cc. of a 1 per cent suspension of this purified product was fed to rats according to the routine method, it was found that it possessed the usual antirachitic potency (Table I). In other words, the activation of the cholesterol does not seem to be due to impurities but to the action of the rays on sterol itself. Moreover, when a watery filtrate was prepared from irradiated cholesterol and tested biologically, it was found to possess no antirachitic potency whatsoever (Table I).

TABLE  
Feeding Experiments with Various Preparations of Cholesterol.

Rat No.	Weight.	Rickets-producing diet.	Substance fed.	Rickets.		
				Radiographic.	Macroscopic.	Microscopic.
10071	40-40	Low phos- phorus No. 84.	0.25 cc. <i>watery filtrate</i> of 1 per cent irradiated choles- terol.	Slight (healing). " Moderate (healing). "	Slight. Moderate. Slight.	Moderate. Slight.
10072	36-32					
10073	40-40					
10112	36-36	" "	0.25 cc. 1 per cent <i>purified</i> <i>cholesterol</i> irradiated $\frac{1}{3}$ hr. at 1 ft.	No. " " "	No. " " "	No. " " "
10113	40-40					
10114	40-40					
10115	40-40					
10116	42-40	" "	0.1 cc. 2.5 per cent <i>anhydrous</i> <i>cholesterol</i> irradiated $\frac{1}{3}$ hr. at 1 ft.	" " " "	" " " "	" " " "
10117	40-40					
10118	36-40					
10119	40-34					
10132	34-39	" "	Controls.	Slight. " " Very slight.	Moderate. " " "	Moderate. " " "
10133	40-44					
10135	40-42					
10134	36-34					

Table I also shows that anhydrous cholesterol can be endowed with antirachitic potency by means of irradiation. The water of crystallization was removed from this preparation by drying at 105°C. *in vacuo* over phosphorus pentoxide.

It may be remembered that in a previous communication it was reported that the saturated reduction products of cholesterol and of phytosterol—dihydrocholesterol and dihydrophytosterol—were not activated by irradiation, that they did not acquire antirachitic properties. Furthermore, spectrograms showed that, in contradistinction to ordinary cholesterol and phytosterol, these products had not been altered in their transmission for ultra-violet rays. These two preparations were tested in order to ascertain whether the action of the ultra-violet rays takes place at the site of the double bond. The negative result which we obtained with these saturated sterols seemed to point in this direction. With this question in mind a cholesterol acetate was prepared by boiling the purified cholesterol with acetic anhydride and recrystallizing the product several times from alcohol. The preparation had the following constants: melting point 113–114°C.;  $[\alpha]_D^{23} = -41.02^\circ$  in chloroform solution. The melting points given above are uncorrected. Cholesterol acetate is an unsaturated compound of cholesterol, the acetate replacing the hydroxyl radicle. As indicated in Table II, this product was found to possess antirachitic potency following irradiation. This positive result, taken in conjunction with the previous failure to activate the saturated sterols, must be regarded as further evidence in favor of the importance of the double bond in enabling this chemical reaction.

An acetate prepared from *irradiated* cholesterol did not differ in appearance, melting point, or specific rotation from the acetate prepared from ordinary cholesterol.

Another link in this chain of evidence is the fact that irradiation, when somewhat prolonged, caused a definite change in the iodine number of the cholesterol. Although irradiation for a period of 2 hours failed to bring about a change in this regard, when this treatment was continued for 6 hours, a definite decrease in iodine absorption resulted; whereas the iodine number was 66.97 to 67.90 previous to irradiation, it was found to have fallen to 56.07 to 56.28 subsequently. In this connection it may



TABLE II.  
*Feeding Experiments with Irradiated Cholesterol Acetate.*

Rat No.	Weight. gm.	Rickets-producing diet.	Substance fed.	Rickets.		
				Radiographic.	Macroscopic.	Microscopic.
10074	30-38	Low phos- phorus No. 84.	0.1 cc. 2.5 per cent cholesterol acetate irradiated dry $\frac{1}{2}$ hr. at 1 ft.	No.	No.	Negative.
10076	30-30			"	"	Slight.
10077	40-40			"	"	Negative.
10078	40-36	" " "	Controls.	R. (?)	Moderate.	Moderate.
10079	40-34			Slight.	"	"
10080	30-28			"	"	"
10082	30-26			"	"	"
10081	44-48			Moderate.	"	"

be added that cholesterol absorbs less bromine as the result of having been subjected to ultra-violet radiations.

As is well known, digitonin exerts a hemolytic action on red blood corpuscles, and cholesterol has the power to inhibit this activity. In view of the fact that our experiments had shown that the chemical properties of cholesterol are altered in various ways as the result of irradiation with ultra-violet light, it seemed worth investigating whether its property of inhibiting the action of digitonin remained unchanged after irradiation (2).

A comparison was made of the effect, in relation to hemolysis, of ordinary cholesterol with that of cholesterol which had been irradiated for periods of  $\frac{1}{2}$ , 2, and 10 hours with the radiations from a mercury vapor lamp, set at the distance of 1 foot. For this purpose a suspension of 1 per cent ethereal solution of cholesterol and 0.1 per cent digitonin was tested on the red cells of the dog and of the sheep. In preliminary titrations it was found that 0.4 cc. of a 0.01 per cent solution of digitonin in saline completely hemolyzed 0.2 cc. of a 5 per cent suspension of red cells; the digitonin was dissolved in 4 per cent alcohol before adding the saline solution. It was found that the rate of speed with which the cholesterol bound the digitonin had been altered as the result of irradiation—that the irradiated cholesterol bound digitonin more readily than ordinary cholesterol, and brought about a comparative delay in its hemolytic action. For example, whereas under controlled and constant conditions ordinary cholesterol allowed complete hemolysis to take place immediately, when cholesterol was used which had been irradiated for 2 hours, complete hemolysis did not occur until after an interval of 4 minutes (Table III). When, however, the cholesterol was irradiated for a prolonged period, for 10 hours, this delay—as the table shows—did not take place and complete hemolysis occurred immediately. In this connection it is of interest to bear in mind that other characteristics acquired through irradiation, for example the antirachitic potency, are lost when the raying is continued for 10 hours, or even for a shorter period. In the above experiments the sterol-digitonin mixtures were incubated for 3 to 8 hours. When the incubation was carried out for 10 hours, it was found that the distinction between ordinary and irradiated cholesterol was lost, except in regard to cholesterol

# 418 Irradiated Cholesterol and Phytosterol. V

which had been rayed for 10 hours, which as a rule continued to allow complete hemolysis to take place immediately.

Table IV shows the result of varying the concentration of sterol rather than the duration of the incubation period. A

TABLE III.

*Effect of Irradiation of Cholesterol (Sterol) on Its Power to Inhibit the Hemolytic Action of Digitonin.*

## A. Variation in Duration of Incubation Period.

Duration of irradiation.	Sterol-digitonide suspension. Incubated.	Duration of incubation.	Final suspensions.	Degree of hemolysis.* (Room temperature.)	Time of hemolysis.
hrs.		hrs.			min. sec.
0	Digitonin 10 cc. of 0.1 per cent (in 0.85 per cent NaCl). Sterol 0.05 cc. of 1 per cent (in ether).	3	Red cells 0.6 cc.	++++	Immediate.
$\frac{1}{2}$			Sterol-digitonide 0.05 cc.	++++	3 4
2			Saline (0.85 per cent) 9.35 cc.	++++	4 2
10				++++	Immediate.
0	" "	6	" "	++++	14 11
$\frac{1}{2}$				++++	20 30
2				++++	23
10				++++	Immediate.
0	" "	8	" "	++++	30 30
$\frac{1}{2}$				++++	210
2				++++	300-600
10				++++	Immediate.
0	" "	10	" "	0	
$\frac{1}{2}$				0	
2				0	
10				++++	"

\* +++++ = complete.

survey of the data of these tests again shows that irradiation of cholesterol enhances its power to inhibit the hemolytic action of digitonin. For example, moderate hemolysis came about in spite of the presence of 0.08 cc. of a solution of ordinary cholesterol, whereas this amount of irradiated cholesterol absolutely

TABLE IV.  
*Effect of Irradiation of Cholesterol (Sterol) on Its Power to Inhibit the Hemolytic Action of Digitonin.*  
*B. Variation in Concentrations of Sterol.*

[illegible]

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prevented hemolysis. Regarded from the view-point of hemolysis it is once more evident that a moderate degree of irradiation, such as an exposure of 2 hours, increases the activity of cholesterol in binding digitonin, but that prolonged periods, for example 10, 16, 20, and 24 hours, have just the opposite effect.

The chemical relationship between activated cholesterol and the natural antirachitic substances, such as cod liver oil, yolk of egg, and bone marrow, is one of prime importance in a consideration of the etiology of rickets. It has been shown repeatedly that when the cholesterol is removed from cod liver oil by means of precipitation with digitonin its antirachitic titer is greatly increased. If the potency of irradiated cholesterol is due to the same substance as that of cod liver oil, one would expect that it likewise would be rendered more potent by subjection to digitonin—that its antirachitic factor would not be precipitated. An experiment was devised to test this hypothesis. Cholesterol was irradiated for the optimum period (2 hours at a distance of 1 foot) and then precipitated with an alcoholic solution of digitonin according to the method of Thaysen (3). The filtrate was evaporated to dryness *in vacuo*, and the white residue taken up and washed with small volumes of ether. The ether solution was then evaporated *in vacuo*. The residue was amorphous and light yellow in color.

This fraction, as indicated in Table V, was fed to a series of rats in order to ascertain its antirachitic potency. The result of the test may be summarized by the statement that the fraction manifested no protective power whatsoever, whereas the non-fractionated cholesterol, which had been irradiated with the same intensity, protected the rats in every instance. This result cannot be explained on the supposition that the activity of the cholesterol was destroyed by contact with digitonin; animal tests carried out to investigate this aspect showed that irradiated cholesterol maintains its potency even after having been subjected to digitonin for a period of 14 hours. It may be added that a comparison of the amount of irradiated cholesterol with the amount of the digitonide precipitate indicated a loss of about 4.5 per cent (by calculation). Recently Beumer (4) carried out chemical tests to ascertain the loss of digitonin-precipitable substance which occurs in cholesterol following irradiation. He

TABLE V.  
*Feeding Experiments with Non-Precipitable Fraction (Digitonin) of Irradiated Cholesterol.*

Rat No.	Weight. <i>gm.</i>	Rickets-producing diet.	Substance fed.	Rickets.		
				Radiographic.	Macroscopic.	Microscopic.
10168	48-54	Low phos- phorus No. 84.	0.1 cc. 2.5 per cent non-pre- cipitable fraction of cho- lesterol irradiated 2 hrs. at 1 ft.	Moderate.	Moderate.	Moderate.
10169	44-50			"	"	"
10171	44-50			Slight.	"	Slight.
10170	42-52					
10172	40-42	" "	0.1 cc. 2.5 per cent cholesterol irradiated 2 hrs. at 1 ft.	No.	No.	No.
10173	42-44			"	"	"
10174	46-44			"	"	"
10175	50-54			" (?)	"	"
10176	50-54	" "		Moderate.	Moderate.	Moderate.
10177	42-50			"	"	Marked.
10178	40-45			"	"	"
10179	40-50			"	"	"

## 422 Irradiated Cholesterol and Phytosterol. V

found the average loss in the precipitates, although varying widely, to be approximately 8.1 per cent when irradiation was carried out for 2 hours. This divergence in results may be accounted for in part by differences in technique or in the constitution of the original cholesterol. When we irradiated cholesterol for 6, instead of 2 hours, the loss was found to have increased to 9 per cent. The experiments cannot be compared from a biological point of view, as Beumer did not test the non-precipitable residue of irradiated cholesterol for its antirachitic power. He merely states that one can take it for granted that the unsaponifiable antirachitic factor is the same substance which is developed from cholesterol as the result of irradiation, and which these experiments have demonstrated to be lost in the course of the precipitation of irradiated cholesterol by means of digitonin.

This experiment does not warrant our concluding that the active principle of cod liver oil and other natural antirachitic substances is essentially different from the active factor of irradiated cholesterol. The marked difference in the menstruum treated with digitonin—the cholesterol solution and the fish oil—makes a comparison of the filtrates unsatisfactory. It should be noted, however, that the amount of the non-precipitable fraction of irradiated cholesterol which was employed in the feeding tests was comparatively large, representing more than 20 times the minimum protective dose of the original irradiated cholesterol. The question of the identity of the specific factor in cod liver oil and that of irradiated cholesterol will have to be studied by other methods before it can be definitely answered.

### CONCLUSIONS.

Purified cholesterol and anhydrous cholesterol can be rendered antirachitic by ultra-violet irradiation, which indicates that it is the sterol which is activated. The watery filtrate of irradiated cholesterol was found to be inactive.

Cholesterol acetate, an unsaturated ester of cholesterol, developed antirachitic potency as the result of irradiation. This must be regarded as additional evidence that the double bond plays an essential rôle in activation.

Irradiated cholesterol showed a greater power to inhibit the hemolytic action of digitonin than the non-irradiated sterol. However, this inhibiting effect is lost when the cholesterol has been irradiated for a prolonged period; a result which is similar to the development and loss of antirachitic potency following irradiation.

A comparison between the digitonin-precipitate of irradiated and non-irradiated cholesterol showed that following irradiation all of the sterol could not be precipitated.

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# A NEW METHOD FOR THE IDENTIFICATION AND ESTIMATION OF CHOLESTEROL AND CERTAIN OTHER COMPOUNDS.

BY JOHN VERNON STEINLE AND LOUIS KAHLENBERG.

(From the Chemical Laboratory, University of Wisconsin, Madison.)

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## INTRODUCTION.

In a previous article<sup>1</sup> the action of cholesterol upon some anhydrous inorganic chlorides and bromides was described and it was stated that cholesterol reacts with antimony pentachloride forming a gummy, chocolate-brown mass. This reaction has been studied further. It forms the basis of the new method to be presented.

When a tiny crystal of cholesterol is treated with a few drops of antimony pentachloride, a dark brown, smeary mass is formed, which dissolves slowly in chloroform. As this chloroform solution is highly diluted with more chloroform, a beautiful cobalt-blue color is developed. The color of this solution is so intense, even at high dilutions, that the reaction at once seemed promising for the detection and colorimetric estimation of small amounts of cholesterol. Two or three drops of a 1 per cent solution of cholesterol in chloroform when treated with a few drops of antimony pentachloride readily form the dark brown precipitate which, upon dilution with more chloroform, slowly dissolves and yields the beautiful blue solution. The blue coloration develops from the brown precipitate under certain definite conditions which were first determined.

### *Antimony Pentachloride Reagent.*

Antimony pentachloride fumes strongly in the air and is consequently a very disagreeable reagent to use. However, it serves equally well when dissolved in chloroform, and such a

<sup>1</sup> Kahlenberg, L., *J. Biol. Chem.*, 1922, lii, 217.

solution is much more convenient. 20 cc. of antimony pentachloride were dissolved in about 80 cc. of chloroform to make 100 cc. of solution. This solution is light yellow in color and does not fume in the air. It will be referred to hereafter as the  $\text{SbCl}_5$  reagent. The  $\text{SbCl}_5$  which must be used in preparing this reagent satisfactorily requires more careful and detailed consideration. The  $\text{SbCl}_5$  first used was a lecture sample which had stood for years in a well stoppered glass bottle. The bottle contained about 100 cc. and there had developed in it a sediment about 1 cm. deep. This was found to be a basic chloride that had probably formed by the action of water on the  $\text{SbCl}_5$ . Since the total amount of  $\text{SbCl}_5$  was rather inadequate for this research, a further supply of "antimonic chloride" was purchased from Merck. The  $\text{SbCl}_5$  reagent (20 cc. of  $\text{SbCl}_5$  to 80 cc. of  $\text{CHCl}_3$ ) was prepared with this new sample as before, but no brown precipitate with cholesterol and no subsequent blue color could be obtained with this new reagent, a reddish brown coloration only being observed. A careful study showed that Merck's sample of  $\text{SbCl}_5$  contained free chlorine, and that this was the reason why the brown precipitate and the blue coloration could not be obtained with it. When chlorine was passed into the original lecture sample of  $\text{SbCl}_5$ , it too no longer yielded the brown and blue compounds; that is to say, it acted exactly like the Merck sample. When Merck's  $\text{SbCl}_5$  was treated with  $\text{SbCl}_3$  to remove the free chlorine, it at once yielded the brown precipitate and the blue coloration. The same was true of the lecture specimen of  $\text{SbCl}_5$  which had been treated with chlorine. *It is consequently necessary that the  $\text{SbCl}_5$  used contain no free chlorine.* Any excess of chlorine present may be removed by aeration; but it is better to dissolve enough  $\text{SbCl}_3$  in the  $\text{SbCl}_5$  to unite with the excess of chlorine present. It was found by independent tests that  $\text{SbCl}_3$  does not develop a color reaction with cholesterol and that consequently the very small amount of it which dissolves in  $\text{SbCl}_5$  has no effect.

#### *Color Reaction.*

On treatment of a chloroform solution of cholesterol with the  $\text{SbCl}_5$  reagent and then diluting with more chloroform, a variety of color changes may be obtained according to the concentrations

used, as is shown by the results of eighteen different experiments presented in Table I.

From Table I it is evident that in only two cases, Experiments 16 and 17, was a final blue color developed. These are cases in which a *cloudy brown* solution was first obtained. This fact agrees with the observation that was originally made; namely, that the blue color develops when the brown mass formed by the

TABLE I.

Experiment No.	Cholesterol solution.*	Dilute with CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.*	Color.	Solution in CHCl <sub>3</sub> .	Final color.
	<i>drops</i>	<i>cc.</i>	<i>drops</i>		<i>cc.</i>	
1	1	0.5	2	Colorless.		
2	1	0.5	8	Pink-orange	1.5	Lighter orange.
3	1	0.5	32	Light orange.	1.5	" "
4	1	0.5	128	Yellow.	2	" yellow.
5	2	0.5	2	Light orange.	1	" orange.
6	2	0.5	8	Orange.	2	Drab.
7	2	0.5	32	Dark orange.	3	Lighter orange.
8	2	0.5	128	Yellow.	3	" yellow.
9	4	0.5	2	Pink-orange.	1.5	Colorless.
10	4	0.5	4	Orange.	3	Light orange.
11	4	0.5	8	Red-brown.	3	Violet.
12	4	0.5	16	Dark brown-red.	3	Orange.
13	4	0.5	32.	Lighter brown.	3	"
14	4	0.5	128	" "	3	Light orange.
15	8	0.5	2	Light "	1	Lighter brown.
16	8	0.5	8	Cloudy "	5	Blue.
17	8	0.5	16	" "	5	Deep blue.
18	8	0.5	128	Red-brown.	5	Lighter brown.

\* Concentration: 1 gm. of cholesterol in 100 cc. of CHCl<sub>3</sub> solution. 80 drops of cholesterol solution in chloroform are equivalent to 1 cc. 60 drops of the SbCl<sub>5</sub> reagent are equivalent to 1 cc.

action of antimony pentachloride on cholesterol is dissolved in chloroform. *The formation of this brown mass is therefore essential to obtain the blue color.* From Table I, Experiments 16 and 17, it is further evident that about 0.1 cc. of the cholesterol solution (0.001 gm. of cholesterol) forms the blue color with 0.1 cc. to 0.2 cc. of the SbCl<sub>5</sub> reagent. The reaction is even more delicate, as will be shown later in connection with the quantitative estimation.

In performing the experiments of Table I, it was observed that an appreciable length of time is required for the cloudy brown precipitate to form after the  $\text{SbCl}_5$  reagent has been added. This matter was therefore investigated and the results are shown in Table II.

From Table II it is apparent that some time is necessary for the formation of the brown precipitate and the subsequent development of the blue color. About 5 minutes appears to be sufficient.

In order to test the delicacy of the reaction further, the experiments presented in Table III were performed.

TABLE II.

Experiment No.	Cholesterol solution.*	Dilute with $\text{CHCl}_3$ .	$\text{SbCl}_5$ reagent.	Time standing.	Color.	Solution in $\text{CHCl}_3$ .	Final color.
	cc.	cc.	cc.	min.		cc.	
1	0.1	0.5	0.2	0	Clear brown.	5	Brown to violet-brown.
2	0.1	0.5	0.2	1	Cloudy "	5	Violet brown to dirty, light blue.
3	0.1	0.5	0.2	2	" "	10	Blue.
4	0.1	0.5	0.2	1	" "	10	Light blue, dark in 1 min.
5	0.1	0.5	0.2	4	" "	10	Violet, turning to blue.
6	0.1	0.5	0.2	5	" "	10	" "
7	0.1	0.5	0.2	7	" "	10	" "
8	0.1	0.5	0.2	10	" "	10	" "

\* Concentration: 1 gm. of cholesterol in 100 cc. of  $\text{CHCl}_3$  solution.

From Table III it appears that as small an amount of cholesterol as 0.000125 gm. will still develop a visible blue color. The first four experiments of Table III would indicate that the reaction might be used for estimating cholesterol quantitatively. Preliminary experiments presented in Table IV show that such is indeed the case. Blue solutions prepared from different amounts of cholesterol in the manner already indicated were carefully compared in a Duboscq colorimeter. A chloroform solution containing 0.25 gm. of cholesterol in 100 cc. was employed. The original solution was diluted with chloroform so as to bring each solution

up to the same volume when the  $\text{SbCl}_5$  reagent was added. 0.2 cc. of the  $\text{SbCl}_5$  reagent was added in each case, and after 5 minutes

TABLE III.

Experiment No.	Cholesterol solution.*	Dilute with $\text{CHCl}_3$ .	$\text{SbCl}_5$ reagent.	Time standing.	Color.	Solution in $\text{CHCl}_3$ .	Final color.
	drops	cc.	cc.	min.		cc.	
1	8	0.5	0.2	5	Cloudy brown.	10	Deep blue.
2	7	0.5	0.2	5	" "	10	Blue.
3	6	0.5	0.2	5	" "	10	Light blue.
4	5	0.5	0.2	5	Slight cloudy brown.	10	Very light blue.
5	5	0.2	0.2	5	Cloudy brown.	10	Light blue.
6	4	0.2	0.2	5	Slight cloudy brown.	10	Very light blue.
7	3	0.2	0.2	5	Clear brown.	10	Visible blue.
8	2	0.2	0.2	5	" "	10	Almost colorless.
9	2	0.0	0.2	5	Slight cloudy brown.	10	Very light blue.
10	1	0.0	0.2	5	Clear brown.	10	Almost colorless.
11	1	0.0	0.1	5	" "	5	Visible blue.

\* Concentration: 1 gm. of cholesterol in 100 cc. of  $\text{CHCl}_3$  solution. 80 drops of cholesterol solution in chloroform are equivalent to 1 cc.

TABLE IV.

Experiment No.	Cholesterol solution.	$\text{CHCl}_3$	Colorimeter readings, scale degrees.					Comparative results. Amount of cholesterol $\times$ colorimeter readings.				
			(b)	(c)	(d)	(e)	(f)	(a $\times$ b)	(a $\times$ c)	(a $\times$ d)	(a $\times$ e)	(a $\times$ f)
	(a)	(g)										
1	0.2	0.4			20.5	23.0	25.0			4.10	4.60	5.00
2	0.3	0.3	20.3	16.9	13.5	15.0	16.3	6.09	5.07	4.05	4.50	4.89
3	0.4	0.2	15.1	12.6	10.0	11.1	12.3	6.04	5.04	4.00	4.44	4.92
4	0.5	0.1	12.0	10.0	7.9	9.0	9.8	6.00	5.00	3.95	4.50	4.90
5	0.6	0.0	10.0	8.3				6.00	4.98			

standing each sample was dissolved in 10 cc. of chloroform. All of the samples were allowed to stand in the light for 5 minutes, after which they were compared in the colorimeter.

Under colorimeter readings, the values printed in bold type are the ones which were used as the standard for comparison with the others. The amounts of cholesterol as represented by "a" were multiplied by each of the colorimeter scale readings in order to show the true comparative values of these readings. The values in Table IV compare very favorably. The more accurate comparisons were, of course, made with the solutions containing higher amounts of cholesterol, Experiments 3, 4, and 5, Table IV.

More complete data on the quantitative nature of this reaction will be presented in later tables. Table IV was inserted at this point in order to show that the reaction is accurately quantitative and to describe the method of comparison which will be used from time to time below in demonstrating other points concerning the reaction.

#### *Composition of the Brown Precipitate.*

The composition of the muddy brown precipitate which is produced when  $\text{SbCl}_5$  acts upon cholesterol, and which upon solution in chloroform yields the blue color, was next determined. This brown substance is non-crystalline, and can only be obtained by using an excess of  $\text{SbCl}_5$  which must, of course, again be removed in purifying this compound. To do this without destroying the brown compound of cholesterol and  $\text{SbCl}_5$  presented great difficulty. It was found that the brown substance is soluble in chloroform and in alcohol and very sparingly soluble in carbon tetrachloride. Cholesterol is soluble in carbon tetrachloride, and the same is true of  $\text{SbCl}_5$ . Therefore the brown precipitate was treated with carbon tetrachloride repeatedly to free it from any uncombined  $\text{SbCl}_5$  or cholesterol. The product remaining was found to dissolve readily in chloroform yielding a clear, beautiful blue solution. Three different samples of the brown compound were prepared and purified in entirely independent experiments. From 2 to 3 gm. were prepared in each case. The procedure was as follows:

About 0.5 gm. of pure, dry cholesterol was treated in a small evaporating dish with either pure antimony pentachloride or a solution of  $\text{SbCl}_5$  in chloroform (10 cc. of  $\text{SbCl}_5$  to 10 cc. of  $\text{CHCl}_3$ ). The antimony pentachloride was added to the cholesterol, a few

drops at a time, until the action had ceased. A dark brown, sticky mass was obtained. The dish was now filled with carbon tetrachloride, and the mass agitated with a glass stirring rod. A pinkish solution containing a fine suspension resulted, leaving the brown mass darker and more cohesive. The liquid was then decanted off, and fresh carbon tetrachloride added. This second amount of the carbon tetrachloride became scarcely colored. The dark gum-like mass was kneaded with the flat end of a stirring rod so as to bring into solution any excess of antimony pentachloride adhering to the compound. This kneading was continued for several minutes. The liquid was then decanted from the material and fresh carbon tetrachloride added. This process was repeated from ten to fifteen times, till no odor of antimony pentachloride could be detected. The material was now almost black in appearance. The gum was then pressed into a thin sheet on a large flat, glass plate, and allowed to dry for 24 to 48 hours. When dry, the material could be scratched from the glass for it was very brittle. The black powder so obtained dissolved readily in chloroform yielding the characteristic blue color already described. *This, therefore, was the purified compound which caused the blue coloration when the  $SbCl_5$  reagent was added to cholesterol and diluted with chloroform.*

An analysis of this compound was made. In analyzing for antimony, the Henz<sup>2</sup> trisulfide method was used with a slight modification in the mode of precipitation. The modified method is briefly as follows:

A weighed amount of the material is dissolved in chloroform. Hydrogen sulfide is passed into the cold solution for half an hour; then without stopping the current of the gas, the solution is heated by placing the precipitation flask in a bath of warm water (50–60°), and hydrogen sulfide is allowed to pass through for another half hour, at the end of which time some more warm chloroform is added to replace that which has evaporated, and the flask placed in boiling water for 5 minutes. The precipitate is allowed to settle, and filtered off in a Gooch crucible which has previously been heated at 280–300°. The precipitate is washed four or five times with chloroform. The remainder of the procedure is identical with the method of Henz. The pentasulfide is converted to

<sup>2</sup> Henz, F., *Z. anorg. Chem.*, 1903, xxxvii, 18.



the black modification of the trisulfide by heating in an atmosphere of carbon dioxide. A special apparatus designed by Henz is used for this treatment. The temperature of the oven is kept at 100–130° for 2 hours, and then raised to 280–300° for 2 hours longer. The crucible is cooled in the stream of carbon dioxide, and weighed.

This modification of the Henz method was first tried out with pure antimony pentachloride in chloroform solution. Very good results were obtained. The Merck "antimonic chloride" was redistilled in a partial vacuum. A fraction passing over at a constant boiling point was used. This method is applicable to organic compounds only when they are soluble in chloroform and contain antimony in a form which is readily split off by hydrogen sulfide.

The data obtained in the analysis of three solutions of antimony pentachloride of known composition are as follows:

<i>Experiment 1.</i>	Weight of $\text{SbCl}_5$ taken	= 0.6514 gm.	= 0.2632 gm. Sb.
	" " $\text{Sb}_2\text{S}_3$ formed	= 0.7356 "	= 0.2627 " "
		Error = 0.0005 "	
<i>Experiment 2.</i>	Weight of $\text{SbCl}_5$ taken	= 0.7034 gm.	= 0.2842 gm. Sb.
	" " $\text{Sb}_2\text{S}_3$ formed	= 0.7947 "	= 0.2838 " "
		Error = 0.0004 "	
<i>Experiment 3.</i>	Weight of $\text{SbCl}_5$ taken	= 0.5864 gm.	= 0.2369 gm. Sb.
	" " $\text{Sb}_2\text{S}_3$ formed	= 0.6622 "	= 0.2365 " "
		Error = 0.0004 "	

The data show that the method is quite satisfactory.

Using this method of analyzing for antimony in the new compound the following data were obtained.

<i>Experiment 1.</i>	Weight of compound taken	= 0.1332 gm.
	" " $\text{Sb}_2\text{S}_3$ formed	= 0.0654 " = 0.0233 gm.
Sb, or 17.49 per cent Sb in the sample.		
<i>Experiment 2.</i>	Weight of compound taken	= 0.1600 gm.
	" " $\text{Sb}_2\text{S}_3$ formed	= 0.0790 " = 0.0282 gm.
Sb, or 17.63 per cent Sb in the sample.		
<i>Experiment 3.</i>	Weight of compound taken	= 0.1669 gm.
	" " $\text{Sb}_2\text{S}_3$ formed	= 0.0815 " = 0.0291 gm.
Sb, or 17.44 per cent Sb in the sample.		

The average result of the three experiments is 17.52 per cent Sb.

The compound was analyzed for chlorine by weighing the chlorine as silver chloride. The material was rendered water-

soluble by careful fusion with a half and half mixture of sodium and potassium carbonates. The fusion was dissolved in water, acidified with nitric acid, and the analysis conducted in the usual manner. The following are the data of three chlorine determinations made.

*Experiment 1.* Weight of compound taken = 0.1374 gm.

“ “ AgCl formed = 0.1434 “ = 0.0354 gm.

Cl, or 25.76 per cent Cl in the sample.

*Experiment 2.* Weight of compound taken = 0.1402 gm.

“ “ AgCl formed = 0.1469 “ = 0.0363 gm.

Cl, or 25.92 per cent Cl in the sample.

*Experiment 3.* Weight of compound taken = 0.1391 gm.

“ “ AgCl formed = 0.1456 “ = 0.0360 gm.

Cl, or 25.89 per cent Cl in the sample.

The average result of the three experiments is 25.86 per cent Cl.

The carbon content was determined by combustion. Lead chromate containing a small amount of potassium dichromate was used in the combustion tube in place of copper oxide. A silver spiral was placed in the end of the tube to insure the retention of the chlorine. The antimony in the compound was thus held as lead antimonate.<sup>3</sup> A small amount of potassium dichromate was mixed with the sample to insure complete combustion. The following are the data obtained.

*Experiment 1.* Weight of compound taken = 0.3421 gm.

“ “ CO<sub>2</sub> formed = 0.3922 “ = 0.1681 gm.

C, or 49.14 per cent C in the sample.

*Experiment 2.* Weight of compound taken = 0.2914 gm.

“ “ CO<sub>2</sub> formed = 0.3290 “ = 0.1410 gm.

C, or 48.39 per cent C in the sample.

The average result of the two experiments is 48.76 per cent C.

No analysis for hydrogen was made. The percentage composition of the compound then is:

	Found.	Computed for C <sub>17</sub> H <sub>16</sub> O·SbCl <sub>5</sub> .
	per cent	per cent
Sb,	17.52	17.63
Cl,	25.86	25.86
C,	48.76	46.07

<sup>3</sup> Morgan, G. T., *Organic compounds of arsenic and antimony*, New York, 1918, 350.

It is evident then that the brown precipitate is a simple addition product consisting of one molecule of cholesterol and one molecule of  $\text{SbCl}_5$ .

An attempt was made to determine the molecular weight of the compound in chloroform solution by the boiling point method. No satisfactory results were obtained, for enough of the compound could not be dissolved in chloroform to cause a sufficient elevation of the boiling point.

The compound, however, is much more copiously soluble in ether, yielding a dark brown solution. The elevation of the boiling point of the ether solution was determined in an ordinary McCoy apparatus. The following data were obtained.

Weight of sample taken.....	= 0.4244 gm.
Rise of boiling point observed.....	= $0.100^\circ$
Volume of solution.....	= 20 cc.

Taking the molecular rise of the boiling point for ether as 3030, and calculating the molecular weight by the usual formula, the result 642.96 is obtained from the above data. The molecular weight as calculated from the formula  $\text{C}_{27}\text{H}_{46}\text{O} \cdot \text{SbCl}_5$  is 683.5.

The agreement of the calculated and observed values is quite close enough to show that the compound is an addition product of one molecule of cholesterol and one of antimony pentachloride.

Similar addition compounds of antimony pentachloride and various aromatic compounds have been described by Hilpert and Wolf,<sup>4</sup> who consider the antimony pentachloride as added directly to the ring compound like water of hydration. Meyer<sup>5</sup> describes analogous compounds with the quinones; and Thomsen<sup>6</sup> has prepared similar ones with the alkaloids.

The composition of the cholesterol-antimony pentachloride addition compound does not change upon solution in chloroform. This was determined by an analysis for antimony and chlorine according to the method described. Some of the compound was dissolved in chloroform, using an amount of the latter insufficient to dissolve all of the dark brown compound. From the clear

<sup>4</sup> Hilpert, S., and Wolf, L., *Ber. chem. Ges.*, 1913, xvi, 2215.

<sup>5</sup> Meyer, K. H., *Ber. chem. Ges.*, 1908, xli, 2568.

<sup>6</sup> Thomsen, T. S., *Oversigt. k. Dansk. Vidensk. Selsk. Forhandl.*, 1911, 41-55; abstracted in *Chem. Zent.*, 1911, i, 1515.

solution the solvent was evaporated at 25° *in vacuo* and the residue was then analyzed. The analysis proved this residue to be the unchanged compound  $C_{27}H_{46}O \cdot SbCl_5$ .

### *Nature of the Blue Solution.*

Further experiments were performed to study the nature of the blue solution in chloroform. During the process of solution of the dark compound,  $C_{27}H_{46}O \cdot SbCl_5$ , in chloroform, the mass disintegrates and the small particles thus formed rise in the chloroform. The color changes from brown through brownish red, to magenta and purple, finally turning to blue. These changes are rapid.<sup>7</sup>

The blue solution shows a very marked Tyndall effect, which is evident from an examination of a dilute chloroform solution of the *purified compound* in a slit ultramicroscope, though distinct particles cannot be observed. However, a blue solution prepared in the *usual manner* by treating a solution of cholesterol with the  $SbCl_5$  reagent produces particles which are visible in the ultramicroscope. These are undoubtedly particles of antimony oxychloride formed by the action of small amounts of water present on the  $SbCl_5$  reagent.

After 1 hour's treatment of the blue solution in the Svedberg and Nichols<sup>8</sup> centrifuge for determination of the size of colloidal particles no effect was noted. This would show that if the compound were "colloidal," the size of the particles is below  $5\mu$ .

An attempt was made to dialyze the blue solution. The membrane employed was a very thin sheet of vulcanized rubber<sup>9</sup> such as is used by dentists. The rubber was stretched over the end of a thistle tube, and secured with silk thread after being reinforced on each side by a piece of very fine white China silk to prevent the rubber from sagging when it became saturated with chloroform. The blue solution was placed on the inside of the thistle tube, and the whole suspended in a small crystallizing dish containing chloroform. After several hours standing, the chloroform on the

<sup>7</sup> While the color changes are rapid when cholesterol and  $SbCl_5$  are used, it will be shown later that when some of the terpenes are substituted for cholesterol, the changes proceed more slowly and in some cases do not even reach the blue stage.

<sup>8</sup> Svedberg, T., and Nichols, J. B., *J. Am. Chem. Soc.*, 1923, xlv, 2910.

<sup>9</sup> Cf., Kahlenberg, L., *J. Physic. Chem.*, 1906, x, 141.

outside showed no signs of turning blue. None of the blue compound passed through the rubber membrane.

A chloroform solution of cholesterol, and a dilute chloroform solution of the  $\text{SbCl}_5$  reagent were each separately dialyzed, using similar rubber membranes. Cholesterol could be detected in the outer liquid within a few minutes after the experiment was started. Antimony pentachloride reacts with the membrane and after about an hour the inner liquid turns brown. However,  $\text{SbCl}_5$  could be detected in the outer liquid by means of hydrogen sulfide within a few minutes after starting the experiment. These experiments could not be conducted over a long period of time for the rubber finally dissolves in the chloroform.

Experiments on dialysis of the blue compound through rubber membranes were carried out on a larger scale. Membranes 5 inches in diameter were used. The dialysis was carried on at  $10^\circ\text{C}$ . in the dark, and the inner liquid was stirred constantly. After an hour, no trace of a blue color could be seen in the outer liquid.

The blue solution shows no absorption lines in the visible spectrum. There is a slight fading at each end of the spectrum, and the yellow and orange are turned to a green.

The analytical data and the results of the molecular weight determination presented above show that the blue color developed in chloroform by the addition of the  $\text{SbCl}_5$  reagent to a cholesterol solution is due to the addition compound of cholesterol and antimony pentachloride,  $\text{C}_{27}\text{H}_{46}\text{O} \cdot \text{SbCl}_5$ . This compound when dissolved in ether yields a true solution whose rise of boiling point was actually determined. A Tyndall effect may indeed be observed in both the ether solution (which is brown) and also in the chloroform solution (which is blue); but the observation of such Tyndall effect does not justify one in concluding that we are dealing with mechanical suspensions rather than with true solutions, for in the ultramicroscope no distinct particles could be discovered; so that if such are present at all, they would have to be smaller than  $5\mu$ .

#### *Quantitative Colorimetric Determinations.*

The following additional data are presented to substantiate further the value of this reaction for the quantitative estimation of cholesterol.

The experiments in Table V were performed as follows: The cholesterol solution used contained 0.25 gm. of cholesterol in 100 cc. of the chloroform solution. The amounts of this solution used are shown in Table V. The solution was not diluted before the addition of the reagent. 0.2 cc. of the  $\text{SbCl}_5$  reagent was used in each case, and the mixtures were allowed to stand exactly 5 min-

TABLE V.

Experiment No.	Cholesterol solution. (a)	$\text{CHCl}_3$ cc.	Colorimeter readings, scale degrees.							
			(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)
	cc.	cc.								
1	0.1	9.9								25.0
2	0.2	9.8						22.4	15.0	12.6
3	0.3	9.7	26.9	23.5	20.1	25.2	20.0	15.0	10.0	8.2
4	0.4	9.6	20.0	17.5	15.0	18.8	15.0	11.2	7.4	6.1
5	0.5	9.5	16.0	14.0	12.0	15.0	14.0	9.0	6.0	4.9
6	0.6	9.4	13.3	11.7	10.0	12.5	9.9	7.4	4.9	
7	0.7	9.3	11.4	10.0	8.6	10.8	8.5	6.3	4.2	
8	0.8	9.2	10.0	8.8	7.6	9.5	7.4	5.7	3.6	
			Comparative results Amounts of cholesterol $\times$ colorimeter readings.							
			(a $\times$ b)	(a $\times$ c)	(a $\times$ d)	(a $\times$ e)	(a $\times$ f)	(a $\times$ g)	(a $\times$ h)	(a $\times$ i)
1										2.50
2								4.48	3.00	2.52
3			8.07	7.05	6.03	7.56	6.00	4.50	3.00	2.46
4			8.00	7.00	6.00	7.52	6.00	4.48	2.96	2.44
5			8.00	7.00	6.00	7.50	6.00	4.50	3.00	2.45
6			7.98	7.02	6.00	7.50	5.94	4.44	2.94	
7			7.98	7.00	6.02	7.56	5.95	4.41	2.94	
8			8.00	7.04	6.08	7.60	5.92	4.56	2.88	

utes, after which they were dissolved in the amount of chloroform indicated, in order that each solution would be of the same volume. All of the tubes were allowed to stand in the light of a 100 watt electric lamp for 5 minutes and then compared. The solutions were all prepared simultaneously and under the same conditions. The columns presenting the product of the amount of cholesterol solution in cc. and the colorimeter scale readings show satisfactory

agreement. The largest errors are found in the comparison of solutions containing widely deviating amounts of cholesterol. Each sample was compared with as many others as possible. The sample used as the standard is printed in bold type.

With the kind assistance of Dr. John R. Koch, unknown solutions of cholesterol were prepared and analyzed. He prepared three solutions of various strengths of cholesterol in chloroform. In each case, a small amount of pure cholesterol was weighed out accurately and dissolved in chloroform. The analysis of the samples is shown in the three experiments which follow.

*Experiment 1.*—The sample was diluted to exactly 10 cc. 0.1 cc. of this solution was treated as in testing for cholesterol by this method. It was

TABLE VI.

Sample No.	Amount.	CHCl <sub>3</sub>	Colorimeter scale degrees. (a)	Amount of cholesterol per 0.1 cc.	Amount of cholesterol in sample. (b)	Scale degrees × weight of cholesterol. (a × b)
	cc.	cc.		gm.	gm.	
1	0.1	9.9	10.9	x	x	10.9 x
Standard.	0.5	9.5	15.0	0.00025	0.00125	0.01875

$$10.9 x = 0.01875$$

Whence  $x = 0.001720$  gm. = weight of cholesterol in 0.1 cc. of solution,  
and  $100 x = 0.1720$  " " in sample diluted to 10 cc.

Weight of cholesterol taken = 0.1731 gm.

" " " found = 0.1720 "

found that a comparable blue color was formed. A fresh sample of the blue solution was then prepared simultaneously and under the same conditions as a blue solution was prepared from a standard solution of cholesterol. The standard solution in chloroform contained 0.25 gm. of cholesterol in 100 cc. The comparison of the samples is shown in Table VI. The same procedure as to reagent, time, etc., was used as in Table V. The difference lies within the limit of experimental error of measuring the sample taken.

*Experiment 2.*—The sample was diluted to 10 cc. as in Experiment 1, but this time 0.1 cc. would not develop a good color. The solution was practically colorless. This meant that the original solution was too dilute. 9 cc. of the sample were then carefully evaporated almost to dryness on a water bath and the residue dissolved to form 1 cc. of chloroform solution. 0.1 cc. of this solution gave a faint blue, but 0.3 cc. gave a good comparable blue color. The same standard and method of comparison were used in this case as in Experiment 1. Table VII gives the results.

*Experiment 3.*—Sample 3 was diluted to 10 cc., and 0.1 cc. treated as in Experiments 1 and 2. The brown precipitate formed was so heavy that it could not be completely dissolved in chloroform, because some of the precipitate adhered to the sides of the test-tube. 1 cc. of this solution was

TABLE VII.

Sample No.	Amount.	CHCl <sub>3</sub>	Colorimeter scale degrees. (a)	Amount of cholesterol per 0.1 cc.	Amount of cholesterol in sample. (b)	Scale degrees × weight of cholesterol. (a × b)
	cc.	cc.		gm.	gm.	
2	0.3	10	19.6	x	3 x	58.8 x
Standard.	0.3	10	15.0	0.00025	0.00075	0.01125

$$58.8 x = 0.01125$$

Whence  $x = 0.000191$  gm. = weight of cholesterol in 0.1 cc. of final solution.

$10 x = 0.00191$  " = weight of cholesterol in 1 cc. of final solution.

$\frac{10}{9} \times 0.0019 = 0.0021$  " = weight of cholesterol in Sample 2.

Weight of cholesterol taken = 0.0023 gm.

" " " found = 0.0021 "

TABLE VIII.

Sample No.	Amount.	CHCl <sub>3</sub>	Colorimeter scale degrees. (a)	Amount of cholesterol per 0.1 cc.	Amount of cholesterol in sample. (b)	Scale degrees × amount of cholesterol. (a × b)
	cc.	cc.		gm.	gm.	
3	0.1	9.9	14.3	x	x	14.3 x
Standard.	0.8	9.2	10.0	0.00025	0.00200	0.02000

$$14.3 x = 0.02000$$

Whence  $x = 0.001398$  gm. = weight of cholesterol in 0.1 cc. of final solution.

$1000 x = 1.3980$  " = weight of cholesterol in original solution.

Weight of cholesterol taken = 1.4005 gm.

" " " found = 1.3980 "

again diluted to 10 cc., and 0.1 cc. of this new, more dilute, solution was treated in the usual manner yielding a deep blue, clear liquid. The same standard and method of comparison were used as in the preceding experiments. Table VIII gives the results.



*Determination of Cholesterol in Blood and Blood Serum.*

Using this method, several experiments were conducted on the determination of cholesterol in ox blood, and ox blood serum. The Myers and Wardell<sup>10</sup> method of extraction was used. The following modification of their procedure to suit the conditions of the new method of estimation was employed.

5 cc. of blood or serum are pipetted into a large porcelain crucible containing 10 to 15 gm. of plaster of Paris, stirred, and dried for an hour in a drying oven. The plaster of Paris is now broken up and placed in a small paper extraction thimble, which is in turn placed in a large weighing bottle, the ends and sides of which have been perforated with a number of small holes. This is now attached to a large cork on a small reflux condenser and the weighing bottle and cork inserted in the neck of a round bottom flask containing 25 to 30 cc. of chloroform. The material is extracted on an electric hot plate for from 1 to 2 hours. The extract is washed out into a small container, evaporated almost to dryness at a low temperature (60–70°C.), and diluted to exactly 5 cc. with chloroform. To 1 cc. of this solution there is added 0.2 cc. of the  $\text{SbCl}_5$  reagent, the mixture is allowed to stand for 5 minutes, and then diluted with 9 cc. of chloroform. The solution is shaken well, and the color allowed to develop for 10 minutes in a strong light. The color is now compared in a Duboscq colorimeter with that of a standard prepared simultaneously and under the same conditions in the following manner. A standard cholesterol solution containing 0.25 gm. of cholesterol in 100 cc. of a chloroform solution is used. To 0.5 cc. of this standard, 0.2 cc. of the  $\text{SbCl}_5$  reagent is added, allowed to stand near the unknown for 5 minutes, and diluted with 9.5 cc. of chloroform. The color is developed in the light beside the unknown, and the comparison then made. Since the extract is diluted to the same volume as the original blood and then 1 cc. of the extract used, the calculation is simple. If the amount of cholesterol in 0.5 cc. of the standard is expressed in mg. (1.25 mg.) the result is obtained directly in mg. of cholesterol per cc. of blood or serum. In blood analysis, the scale reading of the colorimeter is generally 15 degrees. If

<sup>10</sup> Myers, V. C., and Wardell, E. L., *J. Biol. Chem.*, 1918, xxxvi, 147.

so, the following simplified equation may be used,  $x = \frac{18.75}{S}$ , where  $S$  is the scale reading for the unknown and  $x$  expresses mg. of cholesterol per cc. of the unknown.

The blood used in this experiment was fresh ox blood obtained from the Oscar Mayer Packing Company directly after the kill.

Using the above directions, the data in Tables IX and X were obtained.

The results shown here are within the range usually accepted for the cholesterol content of blood. It will be noted that the value

TABLE IX.  
*Blood Serum.*

Sample No.....	1	2	3
Colorimeter scale degrees .....	12.4	12.2	12.6
Mg. of cholesterol per cc. of serum .....	1.51	1.54	1.48

Average of three samples = 1.51 mg. of cholesterol per cc. of serum.

TABLE X.  
*Whole Blood.*

Sample No.....	1	2	3
Colorimeter scale degrees .....	13.0	14.1	13.8
Mg. of cholesterol per cc. of blood.....	1.44	1.33	1.36

Average of three samples = 1.38 mg. of cholesterol per cc. of blood.

for the whole blood is less than that for the blood serum. This was to be expected, since there is a larger percentage of cholesterol in the blood plasma than in the blood corpuscles. The results for the different samples of the whole blood do not check as accurately as the results in the case of the blood serum. This is due to the fact that the extract from the whole blood is slightly colored, and this slight coloration affects the results. It changes the shade of the blue color slightly and makes comparisons less accurate.

The data shown indicate that satisfactory results can be obtained for the estimation of cholesterol in blood by the method described.

Attempts were made to find some blue salt or dye which would prove satisfactory as a standard for comparison of the blue color, and thus obviate the necessity of preparing a fresh standard for each determination. The attempt was unsuccessful since no color of exactly the same shade of blue could be found. This new method is as accurate as any of the methods hitherto in use for the estimation of cholesterol; but it must be admitted that the Liebermann reaction is more delicate.

*Reaction with Phytosterol.*

Phytosterol undergoes the same reaction as does cholesterol when treated with the  $\text{SbCl}_5$  reagent under the same conditions,

TABLE XI.

Experiment No.	Phytosterol solution.*	Dilute with $\text{CHCl}_3$ .	$\text{SbCl}_5$ reagent.	Time.	Color.	Solution in $\text{CHCl}_3$ .	Final color.
	drops	cc.	cc.	min		cc.	
1	2	0.5	0.2	5	Red-orange.	10	Colorless.
2	4	0.5	0.2	5	Muddy brown.	10	Light violet.
3	8	0.5	0.2	5	" "	10	Bluish purple.
4	16	0.5	0.2	5	" "	10	Deep purple-blue.

\* Concentration: 1 gm. of phytosterol in 100 cc. of  $\text{CHCl}_3$  solution. 80 drops of phytosterol solution in chloroform are equivalent to 1 cc.

but produces a solution of a slightly different color. A blue is finally developed, but it takes a longer time. With phytosterol, the color remains in the purplish stage for a longer period. Table XI shows the similarity of the reaction to that of cholesterol.

Colors equivalent in depth are produced by equal amounts of cholesterol and phytosterol. It is very difficult to distinguish between the two by this reaction, but with some practice one may become very apt at recognition of the difference in shade. Indeed, if colors are prepared simultaneously and compared in a colorimeter one can easily note the difference in shade. If the phytosterol color is allowed to develop in a strong light from 10 to 15 minutes longer than that of the cholesterol, the difference is not so easily discerned. It was hoped that a difference between the two might be detected by means of the spectroscope but such was not

the case for neither of these colors showed a dark line absorption in the visible spectrum.

It is evident from Table XI that the formation of a muddy brown precipitate is also a prerequisite for the production of a color with phytosterol. In all respects the behavior of the color due to phytosterol is similar to that of the blue of cholesterol.

Table XII shows that the reaction can also be used for the quantitative estimation of phytosterol. The chloroform solution contained 0.25 gm. of phytosterol in 100 cc. The conditions of the experiments were identical with those used in the experiments shown in Table V, except as to the time of exposure to the 100 watt electric light. The phytosterol color was developed for 10 minutes. The results are as good as those for cholesterol.

TABLE XII.

Experiment No.	Phytosterol solution. (a)	CHCl <sub>3</sub>	Colorimeter readings, scale degrees.					Comparative results Amount of phytosterol × colorimeter readings.				
			(b)	(c)	(d)	(e)	(f)	(a×b)	(a×c)	(a×d)	(a×e)	(a×f)
	cc.	cc.										
1	0.2	9.8			20.5	23.1	<b>25.0</b>			4.10	4.62	<b>5.00</b>
2	0.3	9.7	20.2	16.7	13.5	<b>15.0</b>	16.4	6.06	5.01	4.05	<b>4.50</b>	4.92
3	0.4	9.6	15.0	12.3	<b>10.0</b>	11.1	12.2	6.00	4.92	<b>4.00</b>	4.44	4.88
4	0.5	9.5	12.1	<b>10.0</b>	8.1	9.0	9.8	6.05	<b>5.00</b>	4.05	4.50	4.90
5	0.6	9.4	<b>10.0</b>	8.2				<b>6.00</b>	4.92			

#### *Comparison of Cholesterols and Phytosterols from Different Sources.*

In the preceding experiments, the cholesterol solutions used were prepared from a very fine looking sample purchased from Pfanstiehl; the phytosterol was the purest sample of wheat sitosterol prepared in this laboratory by Dr. J. R. Koch under Professor Kahlenberg's direction. Different samples of cholesterol and phytosterol on hand in the laboratory were now subjected to this test. The depth of the blue colors produced by these samples as measured in the colorimeter indicated their relative purity.

Tables XIII and XIV show the comparative values in the colorimeter of the chloroform solutions of 1 gm. of these sterols in

TABLE XIII.

Sample No.	Source of cholesterol.	Prepared by.	Appearance.	Melting point. °C.	Colorimeter scale degrees.
1	?	Pfanstiehl.	Fine crystals.	148.5	20.0
2	?	Hollister-Wilson.	Needles.	148.5	18.2
3	?	Kahlbaum.	Fine crystals.	148.45	19.9
4	?	Merck.	Slight yellow.	147.95	21.1
5	Pig brain.	Steinle.*	Fine needles.	148.4	19.2
6	Spinal cord of pig.	Kahlenberg.	Flat plates.	148.5	19.5
7	Lanolin.	Steinle.*	Gray flakes.	148.35	19.5
8	Gall-stones.	"	Fine crystals.	148.5	19.5
9	Cod liver oil.	H. Rasmussen.*	"	145.4	23.2
10	Whale oil.	J.C. S. Chow.*	Flakes.	148.0	21.0
11	Wisconsin fish oil.	" " "	Long yellow crystals.	141.6	26.1

\* These men are students who prepared the samples under the direction of Professor Kahlenberg.

100 cc. 0.1 cc. of the solutions was used, and all samples were treated in the usual manner for the preparation of a standard.

The more intense blue given by the Hollister-Wilson sample cannot be explained. Tables XIII and XIV show that the standard used in any colorimetric analytical work is very important and may greatly affect the results obtained. For instance, it is

TABLE XIV.

Sample No.	Source of phytoesterol.	Prepared by.	Appearance.	Melting point.	Colorimeter scale degrees.
				°C.	
1	Wheat oil.	J. R. Koch*	Needles.	136.8	15.0
2	Rye oil.	A. Ludden.*	"	135.5	15.5
3	Oat "	B. Hubbard.*	Yellow needles.	130-133	17.2
4	Peanut oil.	H. Rasmussen.*	Needles.	135-137	15.3
5	Hickory nut oil.	H. D. Royce.*	Fine needles.	132.6	16.7

\* These men are students who prepared the samples under the direction of Professor Kahlenberg.

TABLE XV.

Experiment No.	Chloride in $\text{CHCl}_3$ .	Result with cholesterol.
1	$\text{SbCl}_3$	No action.
2	$\text{AsCl}_3$	" "
3	$\text{PCl}_3$	" "
4	$\text{SiCl}_4$	" "
5	$\text{TiCl}_4$	Cloudy yellow solution.
6	$\text{SnCl}_4$	Yellow color.
7	$\text{SeOCl}_2$	" "
8	$\text{CrO}_2\text{Cl}_2$	No action.

apparent from Table XIII that if the Hollister-Wilson sample had been used as the standard in the blood analysis in Tables IX and X, the results would have been quite different.

#### *Substitution of Other Chlorides for Antimony Pentachloride.*

The action of a number of anhydrous inorganic chlorides on cholesterol has been studied by Kahlenberg.<sup>1</sup> These experiments

were all carried out with the pure chlorides, and since antimony pentachloride was among those used, it was thought advisable to repeat the work using chloroform solutions of these other chlorides. No important new facts were gained, nevertheless, the results of these experiments are given in Table XV. Only in a few cases are colors formed, and in these it is merely a yellow such as is produced by the same reagent with many other organic compounds. About 20 cc. of the anhydrous chloride to 80 cc. of chloroform were used in each experiment. The usual chloroform solution of cholesterol containing 1 gm. in 100 cc. was employed. Different amounts of the various reagents with varying amounts of cholesterol were used.

*Substitution of Other Solvents for Chloroform.*

Various common inorganic solvents were substituted for chloroform in preparing the  $\text{SbCl}_5$  reagent and dissolving the resulting compound. These experiments were performed to ascertain whether or not the color reaction in question depends upon the specific nature of chloroform. Solutions of 20 cc. of antimony pentachloride in 100 cc. of the resulting solution were made with each solvent. The reaction with the solvent was noted. If a satisfactory reagent was formed, it was used in place of the chloroform solution in the cholesterol reaction and the results noted. The solvent in question was, of course, used in each case to dissolve the precipitates formed. In all of the previous experiments Mallinckrodt's U.S.P. chloroform was used. The experiments listed below show the results with different solvents.

*Anhydrous Chloroform.*—The reaction was identical with that of Mallinckrodt's U. S. P. chloroform.

*Carbon Tetrachloride (Mallinckrodt).*—When  $\text{SbCl}_5$  was mixed with this moist  $\text{CCl}_4$  a heavy white precipitate of antimony oxychloride was formed which partially dissolved on standing. On addition of this clear reagent to the cholesterol solution, the usual heavy red-brown precipitate was formed, which dissolved but slightly in additional  $\text{CCl}_4$ , yielding a salmon-pink solution, which faded on standing.<sup>11</sup> Hilpert and Wolf<sup>4</sup> used a carbon tetrachloride solution of  $\text{SbCl}_5$  in studying the color reactions produced by antimony pentachloride with certain aromatic compounds.

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<sup>11</sup> This red-brown precipitate is  $\text{C}_{27}\text{H}_{46}\text{O}.\text{SbCl}_5$ , already described. Dissolved in chloroform it yields the blue solution.

**Carbon Tetrachloride Dried with  $\text{CaCl}_2$ .**—A slight white precipitate of antimony oxychloride was still formed with  $\text{SbCl}_5$ , but it almost entirely redissolved. A deeper pink solution was formed with cholesterol in this case.

**Ethyl Alcohol (55 Per Cent).**—A clear solution was formed with  $\text{SbCl}_5$ , but no reaction took place when added to a cholesterol solution.

**Anhydrous Ethyl Alcohol.**—The same as with the 95 per cent alcohol.

**Methyl Alcohol.**—The results were similar to those with ethyl alcohol.

**Anhydrous Ether.**— $\text{SbCl}_5$  reacts with ether yielding a brown coloration which persists on dilution with ether.

**Acetone.**—Acetone reacts violently with  $\text{SbCl}_5$  forming a dark brown solution.

**Benzene (Kahlbaum).**—Benzene forms a light brown solution with  $\text{SbCl}_5$ , which with cholesterol yields a cloudy, orange precipitate. Upon dissolving this in more benzene, a dirty yellowish green solution is obtained.

**Toluene.**—The reaction is similar to that with benzene.

**Acetic Anhydride.**— $\text{SbCl}_5$  undergoes a violent reaction with acetic anhydride, yielding a dark brown solution.

**Phosphorus Trichloride.**— $\text{PCl}_3$  and  $\text{SbCl}_5$  react violently to form a white precipitate. This white precipitate is a mixture of  $\text{SbCl}_5$  and  $\text{PCl}_5$  formed according to the following equation:



These experiments suffice to show that in the formation of the blue color, chloroform is necessary, and that its action is highly specific.

It has been noted that *the muddy brown precipitate* (which is a prerequisite for color formation) *is obtained only when chloroform or carbon tetrachloride is used as solvent for the  $\text{SbCl}_5$  employed.* The precipitate is  $\text{C}_{27}\text{H}_{46}\text{O} \cdot \text{SbCl}_5$ . This brown precipitate (formed in either chloroform or carbon tetrachloride) on being dissolved in chloroform forms a *blue*, in carbon tetrachloride a *salmon-pink*, and in ethyl alcohol a *bright green* solution. In carbon tetrachloride, the precipitate is but sparingly soluble, as already stated.

The pink formed with carbon tetrachloride is not a striking color, nor is it suitable for quantitative comparison. The green solution in alcohol can be used for colorimetric work but the color is not as deep as the blue obtained with chloroform, nor is the

<sup>12</sup> This equation was verified by actual experiments. The reaction also takes place in chloroform solution, and in both cases the precipitation is instantaneous although the reacting mixture does not conduct electricity. For similar experiments on instantaneous precipitation in non-conducting solutions see Kahlenberg, L., *J. Physic. Chem.*, 1902, vi, 1.



reaction as delicate. There is therefore no advantage in using either carbon tetrachloride or alcohol in place of chloroform for the colorimetric determination of cholesterol.

The behavior of the purified compound  $C_{27}H_{46}O \cdot SbCl_5$  in various solvents is shown in Table XVI.

Table XVI shows that when no excess of  $SbCl_5$  is present the action of some of the common solvents is altered. This is especially true in the case of ether, which reacts strongly with free  $SbCl_5$ .

In a few instances when a large excess of dry cholesterol was used with a small amount of the  $SbCl_5$  reagent, a purple, smeary

TABLE XVI.

Experiment No.	Solvent.	Result.
1	$CHCl_3$	Deep blue solution.
2	$CCl_4$	No action.
3	Acetone.	Very slight solution, light green.
4	$C_2H_5OH$	Slight white precipitate.
5	$CH_3OH$	" " "
6	Benzene.	Slow solution to dark green.
7	Toluene.	Slight " light green.
8	Fractol.*	No action.
9	Ether.	Very soluble, rich brown.
10	$CS_2$	Very slow solution, deep blue green.
11	Water.	White precipitate in time.
12	Acids.	No action.

\* This is a low boiling fraction of gasoline.

mass was formed which readily dissolved in both carbon tetrachloride and in chloroform, yielding a deep purple solution. This is probably a compound of cholesterol and  $SbCl_5$  in different proportions than in the blue one. This purple compound could not be prepared pure enough for analysis, since no solvent for its separation from the excess of  $SbCl_5$  or excess of cholesterol could be found.

#### *Action of the $SbCl_5$ Reagent with Fats, Oils, Etc.*

Some idea of the reaction of the  $SbCl_5$  reagent with fats and oils which contain sterols is essential, so that one may judge as to what

extent fats and other impurities associated with the sterols affect the color. To this end a number of cholesterol- and phytosterol-containing oils and fats, as well as oils free from sterols were tested

TABLE XVII.

Sample No.	Oil.	Color produced.	Change in light.
1	Corn.	Brown.	No change.
2	Sesame.	"	Drab.
3	Castor.	"	Reddish.
4	Olive.	"	No change.
5	Raw linseed.	"	" "
6	Rape seed.	"	" "
7	Cottonseed.	"	Violet-blue.
8	Brazil nut.	"	Dirty light blue.
9	Peanut.	"	No change.
10	Rye.	"	Violet hue.
11	Wheat.	"	" "
12	Hemp seed.	"	No change.
13	Oat.	"	" "
14	Poppy.	"	" "
15	Hickory nut.	"	Slight dirty blue.
16	Soy bean.	"	Violet hue.
17	Hydrogenated cottonseed.	Pink.	Darker pink.
18	Hydrogenated soy bean.	Dark pink.	Magenta.

TABLE XVIII.

Sample No.	CHCl <sub>3</sub> solution of material.	Color produced.	Change in light.
1	Stearic acid.	Slight light yellow.	No change.
2	Palmitic "	Light yellow.	Slightly darker.
3	Oleic "	Red-brown.	Dark red-brown.
4	Linoleic "	"	Purplish.
5	Caprylic "	No color.	No color.
6	Sodium oleate.	Brown.	Darker.
7	Glycerol.	Slight emulsion.	No change.

with the reagent. Other fat-like bodies, fatty acids, and materials which might possibly contaminate the sterols were also treated with the SbCl<sub>5</sub> reagent. The reactions of some oils of vegetable origin are shown in Table XVII.

The experiments with each of the oils were carried on by treating a few drops of the oil with the  $\text{SbCl}_5$  reagent, allowing it to stand, and then diluting with chloroform.

The hydrogenated oils did not act like the unhydrogenated products, for they failed to produce the original brown coloration. From this one might expect that the brown color is due to unsaturated fatty acids in the oil. This is undoubtedly the case as appears from the action of the  $\text{SbCl}_5$  reagent on some of the fatty

TABLE XIX.

Sample No.	Material.	Color produced.	Change in light.
1	Cod liver oil.	Violet.	Blue.
2	Whale oil.	"	Purple.
3	Menhaden oil.	" to purple.	Blue.
4	Wisconsin fish oil.	"	Greenish.
5	Lanolin.	"	Dark blue.
6	Mutton tallow.	Pink.	Deeper pink.
7	Beef "	Yellow.	" yellow.
8	Butter.	Orange-brown.	Darker.
9	Lard.	Light brown.	Deeper brown.

TABLE XX.

Sample No.	Material.	Color produced.	Change in light.
1	Fractol.	Slight yellow.	No change.
2	Stanolax.	Reddish.	Magenta.
3	Mobile A.	Green.	Darker.
4	Paraffin.	Colorless.	Colorless.
5	Vaseline.	Brown.	Violet.

acids (Table XVIII). This test might be applied to ascertain whether or not an oil has been hydrogenated.

The saturated acids do not give the coloration which the unsaturated ones do. Glycerol gives no coloration. The lower fatty acids, like caprylic, give less coloration than do the higher ones.

The fish oils are all high in cholesterol content, and show the cholesterol reaction in spite of the impurities present. The animal oils and fats in general contain less of the unsaturated fatty

acids than the vegetable oils, and therefore do not produce the same brown color which the latter exhibit (Table XIX).

The last four animal fats, which contain but little cholesterol, give no characteristic reaction. Butter and lard containing more of the unsaturated fatty acids give more of a brown coloration.

Marcusson<sup>13</sup> claims that petroleum contains some cholesterol. Several mineral oils originating from petroleum were tested with the  $\text{SbCl}_5$  reagent to see whether or not they produced any color (Table XX).

TABLE XXI.

Sample No.	Material.	Color produced.	Change in light.
1	Isocholesterol.*	Violet blue.	Blue.
2	Cholesterol benzoate. (Schuchardt).	Blue.	Deep blue.
3	Glycocholic acid.*	Greenish.	Darker.
4	Taurocholic " †	Insoluble in $\text{CHCl}_3$ .	No action.
5	Lecithin.‡	Slight orange-pink.	" change.
6	Cephalin.	Very slight dirty yellow.	" "

\* A small amount prepared from lanolin by the method of Schulze, E., *J. prakt. Chem.*, 1873, series 2, vii, 163.

† Glycocholic and taurocholic acids were kindly furnished by Professor Steenbock of the Department of Agricultural Chemistry.

‡ The samples of lecithin and cephalin were prepared by Sarah H. Vance under Professor Kahlenberg's direction and were of a very high degree of purity.

The reactions with stanolax and with vaseline might lead one to suppose that they contain either cholesterol or some cholesterol-like body.

Table XXI shows the reaction with some fat-like bodies and several compounds similar to cholesterol.

Isocholesterol and cholesterol benzoate react the same as cholesterol. The cholic acids, though very similar to cholesterol in composition, do not show the cholesterol reaction. The lipoids, lecithin and cephalin, do not react with the  $\text{SbCl}_5$  reagent.

From the reactions of these various oils and other substances with the  $\text{SbCl}_5$  reagent, it is clear that the sterols must be free from

<sup>13</sup> Marcusson, J., *Chem.-Ztg.*, 1908, xxxii, 391.

all impurities, especially from unsaturated fatty acids, in order that the reaction may be used for quantitative estimations.

A few of the essential oils were tested with the  $\text{SbCl}_5$  reagent (Table XXII).

TABLE XXII.

Sample No.	Material.	Color produced.	Change in light.
1	Oil of lemon.	Brownish violet.	Bluish.
2	" " orange.	Violet.	Dirty blue.
3	Artificial oil of sassafras.	Purple.	Brown.
4	Oil of cedar.	Dark magenta.	Purple.
5	Wild crab-apple wax.	Bright red to magenta.	" to blue.

TABLE XXIII.

*Pinene: C<sub>10</sub>H<sub>16</sub>. B. P. 155.5°.*

Experiment No.	Concentration per 100 cc. $\text{CHCl}_3$ solution	Amount.	Dilute with $\text{CHCl}_3$ .	$\text{SbCl}_5$ reagent.	Solution in $\text{CHCl}_3$	Color.	Final color in light.
	cc.	drops	cc.	cc.	cc.		
1	100	1	0.5	0.2	100	Dark violet.	Blue.
2	100	1	1.0	0.2	100	" "	"
3	100	1	1.5	0.2	75	" red-violet.	"
4	100	1	2.0	0.2	75	Red-violet.	"
5	100	1	2.5	0.2	50	Reddish.	"
6	100	1	1.0	0.2	50	Violet.	Good blue.
7	25	1	0.5	0.2	10	Dark violet.	Dark "
8	12.5	1	0.5	0.2	10	Violet.	Blue.
9	10	1	0.5	0.2	10	Lavender to violet-blue.	"
10	6.25	1	0.5	0.2	10	Light violet.	Faint blue.

The colors produced by the action of the  $\text{SbCl}_5$  reagent on these non-sterol-containing oils is similar to the action of the sterols. These oils, however, contain terpenes. Because of the colors produced by these oils, the action of the  $\text{SbCl}_5$  reagent on some of the common terpenes was studied. It must be remembered that cholesterol and the terpenes have similarity in their structure.

*Reaction with the Terpenes and Allied Substances.*

In Tables XXIII to XL, the concentration of the terpenes<sup>14</sup> used is expressed in cc. (or gm. for solids) of the terpene in 100 cc. of a chloroform solution. It takes 80 drops of the solution to make 1 cc. The reaction with the individual terpenes is first described and then these results are correlated.

Pinene produces the blue color even more slowly than phytosterol. In both cases, the reaction is accelerated by light. However, the blue of the pinene reaction is never quite of the same shade as that of the cholesterol. Experiment 6 (Table XXIII) compares most favorably with the cholesterol standard.

TABLE XXIV.  
*Limonene: C<sub>10</sub>H<sub>16</sub>. B. P. 175.5°*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute with CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution in CHCl <sub>3</sub> .	Color.	Final color in light.
	cc.	drops	cc.	cc.	cc.		
1	100	1	0.5	0.2	30	Deep red-violet.	Purple to blue. Very light blue.
2	1	8	0.5	0.2	10	Pink.	
3	1	16	0.5	0.2	10	Pink-violet.	Violet-blue.
4	1	20	0.5	0.2	10	Violet.	Blue.

Limonene develops the blue color faster than pinene; nevertheless, it takes about twice as long as cholesterol. Experiment 4 (Table XXIV) is comparable in hue with the cholesterol standard.

Thymol is not a true terpene, but a phenol derivative. It contains a benzene ring. It does not undergo the color reaction, but in concentrated solutions it gives a purple color with the SbCl<sub>5</sub> reagent.

Cavacrol is similar to thymol in both composition and reaction with the SbCl<sub>5</sub> reagent.

<sup>14</sup> The samples of the terpenes used in this part of the research were kindly furnished by Dr. Roland Kremers of the Department of Pharmacy of the University of Wisconsin. The samples were of a high degree of purity, and were for the greater part samples prepared by Dr. Kremers personally.

For equal concentrations, menthol and cholesterol give tests which are comparable in depth of color, but the color produced by menthol never reaches the final blue color which cholesterol produces. Even prolonged standing in a strong light will not

TABLE XXV.  
*Thymol*:  $C_{10}H_{14}O$ . M. P.  $44^{\circ}$ .

Experiment No.	Concentration per 100 cc. $CHCl_3$ solution.	Amount.	Dilute with $CHCl_3$ .	$SbCl_5$ reagent.	Solution in $CHCl_3$	Color.	Remarks.
	gm.	cc.	cc.	cc.	cc.		
1	0.1	0.1	0.5	0.2	5	Colorless.	Before solution was purple.
2	0.1	0.5	0 0	0.2	5	Yellow.	
3	1.0	0.5	0.0	0.2	5	Brown.	
4	10.0	0.5	0.0	0.2	5	"	Before solution gives a purple on side of tube; green in bottom.
5	Saturated.	0 5	0.0	0.2	5	"	

TABLE XXVI.  
*Cavacrol*:  $C_{10}H_{14}O$ . B. P.  $236^{\circ}$ .

Experiment No.	Concentration per 100 cc. $CHCl_3$ solution.	Amount.	Dilute with $CHCl_3$ .	$SbCl_5$ reagent.	Solution in $CHCl_3$	Color.	Remarks.
	cc.	cc.	cc.	cc.	cc.		
1	100	0.1	0.5	0.2	10	Orange.	Dark brown.
2	100	0.1	0 1	0 2	10	Red-orange.	Clear color before solution.

cause this change. The color changes to a dark purple at first then fades after an hour or so of exposure.

Borneol will not produce a strong blue color in as dilute solutions as will cholesterol. In Experiment 9 (Table XXVIII), the color developed compares favorably with that of the cholesterol

standard. In order to produce the final blue, the borneol solution must be exposed to a strong light for from 5 to 10 minutes.

TABLE XXVII.  
*Menthol: C<sub>10</sub>H<sub>18</sub>OH. M. P. 43°.*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute with CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution in CHCl <sub>3</sub> .	Color.	Final color in light.
	gm.	cc.	cc.	cc.	cc.		
1	1	0.2	0.0	0.4	10	Magenta.	Purple to purple-blue.
2	1	0.2	0.0	0.2	10	Violet.	" "
3	1	0.25	0.25	0.2	10	Deep magenta.	" "
4	1	0.2	0.5	0.2	10	Light "	Dark violet to purple.
5	1	0.1	0.5	0.2	10	Pink-violet.	Light violet to light purple.

TABLE XXVIII.  
*Borneol: C<sub>10</sub>H<sub>17</sub>OH. M. P. 206.5°*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute with CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution in CHCl <sub>3</sub> .	Color.	Final color in light.
	gm.	cc.	cc.	cc.	cc.		
1	1	0.1	0.5	0.2	5	Light violet.	Darker violet.
2	1	0.2	0.5	0.2	7	Violet.	Violet-blue.
3	1	0.2	0.5	0.4	10	Dirty violet.	Darker violet.
4	1	0.4	0.5	0.2	5	Light brown.	Violet.
5	1	0.2	0.25	0.4	10	" violet.	Darker violet.
6	1	0.2	0.0	0.2	10	" "	" "
7	1	0.2	0.0	0.4	10	Dirty "	" "
8	5	0.2	0.0	0.2	10	Purple.	Dark blue.
9	5	0.2	0.5	0.2	10	Red-violet.	Light "
10	5	0.25	0.25	0.2	10	Magenta.	Green-blue.
11	5	0.4	0.1	0.2	10	Red.	Blue.

The color produced by borneol is probably more nearly comparable to the cholesterol blue than the colors produced by any of the other terpenes.



No final clear blue is obtained with terpeneol, but instead a dirty grayish blue. Experiment 2 (Table XXIX) corresponds to the cholesterol standard in depth of color.

Dilute solutions of camphor will not give a color test under any conditions. Only a saturated solution, which consists of

TABLE XXIX.

*Terpineol: C<sub>10</sub>H<sub>17</sub>OH. M. P. 35°*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute with CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution in CHCl <sub>3</sub> .	Color.	Final color in light.
	cc.	drops	cc.	cc.	cc.		
1	100	1	0 5	0 2	50	Violet.	Dirty violet-blue.
2	10	1	0.5	0 2	10	Magenta.	Purple to dirty blue.
3	5	1	0.5	0.2	10	Red-violet.	Light dirty blue.

TABLE XXX.

*Camphor: C<sub>10</sub>H<sub>16</sub>O. M. P. 175°.*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution of CHCl <sub>3</sub> .	Color.	Final color in light.
	gm.	cc.	cc.	cc.	cc.		
1	.1	0.5	0	0.2	5	Colorless.	Colorless.
2	5	0.5	0	0.4	10	Pink-violet.	"
3	5	0.2	0	0.2	10	Colorless.	"
4	5	0.5	0	0.8	10	Pink.	"
5	10	0.5	0	0.4	10	Deep pink.	Light pink.
6	Saturated.	0.3	0	0.4	10	" violet.	" blue.

about 90 gm. of camphor to 10 cc. of chloroform, will yield any color. A final blue is obtained by exposure to light, but even then it is not as dark as the cholesterol standard. Since such an excessive concentration is necessary to produce the color, it may be that the color produced is due not to the camphor, but rather

to some impurity present. However, the color is produced in the same concentrations by different samples of camphor.

In dilute solutions, thujone has a tendency to form a dirty blue, but never a clear blue like that produced by cholesterol.

Pulegone does not give the cholesterol color test, but forms a permanent red-brown coloration.

TABLE XXXI.

*Thujone: C<sub>10</sub>H<sub>16</sub>O. B. P. 84.5°.*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution of CHCl <sub>3</sub> .	Color.	Final color in light.
	g.	cc.	cc.	cc.	cc.		
1	100	0.1	0.5	0.2	15	Red-brown.	Dirty violet.
2	1	0.1	0.5	0.2	5	Colorless.	Colorless.
3	1	0.5	0.0	0.2	10	Light dirty violet.	Light dirty blue.
4	1	0.6	0.0	0.3	10	Dirty violet.	Dirty blue.
5	2	0.5	0.0	0.2	15	Violet.	Slow fading.

TABLE XXXII.

*Pulegone: C<sub>10</sub>H<sub>16</sub>O. B. P. 121.5°.*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution of CHCl <sub>3</sub> .	Color.	Final color in light.
	g.	cc.	cc.	cc.	cc.		
1	100	0.1	0.5	0.2	20	Red-brown.	No change.
2	1	0.1	0.5	0.2	5	Yellow-orange.	" "
3	1	0.5	0.0	0.2	10	Red-orange.	" "

Cineol gives a poor test after standing in the light for a short time. The blue is never clear.

Anethol gives no satisfactory test.

Eugenol is not a terpene, but a methoxyphenol. The SbCl<sub>5</sub> reagent forms a sticky brown precipitate with it, which is only sparingly soluble in chloroform, and which adheres to the side of the test-tube. No satisfactory color reactions are obtained.

Guaiacol is not a terpene. It does not even contain ten carbon atoms to the molecule. It gives no test which in any way resembles the cholesterol test. It forms a beautiful green solution with the  $\text{SbCl}_5$  reagent. A similar color is formed in an alcoholic solution of guaiacol with ferric chloride.<sup>15</sup>

The linalool molecule contains ten carbon atoms and belongs to the olefinic terpene or terpenogen group. It is, however, a

TABLE XXXIII.  
*Cineol: C<sub>10</sub>H<sub>18</sub>O. B. P. 176°.*

Experiment No.	Concentration per 100 cc. $\text{CHCl}_3$ solution.	Amount.	Dilute with $\text{CHCl}_3$ .	$\text{SbCl}_5$ reagent.	Solution in $\text{CHCl}_3$ .	Color.	Final color in light.
	cc.	drops	cc.	cc.	cc.		
1	100	1	0.5	0.2	30	Dark violet.	Gray-blue.
2	1	16	0.5	0.2	10	Red-violet.	Purple to gray-blue.
3	1	8	0.5	0.2	10	Pink.	Violet to gray-blue.

TABLE XXXIV.  
*Anethol: C<sub>10</sub>H<sub>12</sub>O. B. P. 232°*

Experiment No.	Concentration per 100 cc. $\text{CHCl}_3$ solution.	Amount.	Dilute with $\text{CHCl}_3$ .	$\text{SbCl}_5$ reagent.	Solution in $\text{CHCl}_3$ .	Color.	Final color in light.
	cc.	cc.	cc.	cc.	cc.		
1	100	0.1	0.5	0.2	40	Violet.	Darker.
2	1	0.5	0.0	0.2	10	"	Dirty blue.

branched chain compound and gives no characteristic reaction with the  $\text{SbCl}_5$  reagent, but reacts like an unsaturated fatty acid.

Table XXXVIII has been compiled to give a general summary of the behavior of these various compounds. It shows whether the compounds are menthane, camphane, or benzene derivatives. It also indicates whether they are hydrocarbons or some deriva-

<sup>15</sup> von Richter, V., Organic chemistry, translated by D'Albe, E. E. F., Philadelphia, 1922, ii, 213.

TABLE XXXV.  
*Eugenol: C<sub>10</sub>H<sub>12</sub>O<sub>2</sub>. B. P. 247°.*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute with CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution in CHCl <sub>3</sub> .	Color.	Final color in light.
	cc.	cc.	cc.	cc.	cc.		
1	100	0.1	0.5	0.2	50	Violet.	Deeper violet.
2	1	0.1	0.5	0.2	10	Yellow.	No change.
3	1	0.5	0.0	0.2	10	Brown.	Violet.
4	10	0.5	0.0	0.2	30	Purple.	Purple-blue.
5	10	0.1	0.5	0.2	10	Red.	Purple.
6	10	0.1	0.0	0.2	15	Violet.	Deeper violet.

 TABLE XXXVI.  
*Guaiacol: C<sub>7</sub>H<sub>8</sub>O<sub>2</sub>. B. P. 205°*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute with CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution in CHCl <sub>3</sub> .	Color.
	cc.	cc.	cc.	cc.	cc.	
1	100	0.1	0.5	0.2	50	Green precipitate to green color.
2	2	0.5	0.0	0.2	15	Olive green.
3	2	0.1	0.5	0.2	10	Brown, no precipitate.

 TABLE XXXVII.  
*Linalool: C<sub>10</sub>H<sub>17</sub>O<sub>4</sub>. B. P. 198°*

Experiment No.	Concentration per 100 cc. CHCl <sub>3</sub> solution.	Amount.	Dilute with CHCl <sub>3</sub> .	SbCl <sub>5</sub> reagent.	Solution in CHCl <sub>3</sub> .	Color.	Final color in light.
	cc.	cc.	cc.	cc.	cc.		
1	100	0.1	0.5	0.2	50	Brown.	No change.
2	10	0.1	0.5	0.2	10	Red-brown.	" "

tive, and gives the concentration necessary to develop the color mentioned.

All of the terpenes and terpene-like bodies studied undergo some color reaction with the SbCl<sub>5</sub> reagent, but only three of this list

TABLE XXXVIII.

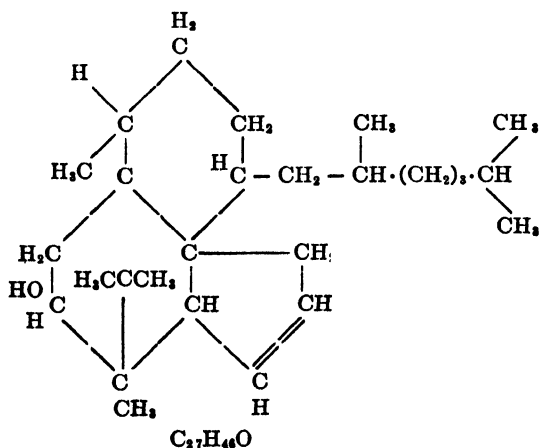
Experi- ment No.	Compound.	Formula.	Group.	Class.	Concen- tration.*	Results.
1	Pinene.	$C_{10}H_{16}$	Terpene.	Hydrocarbon.	10	Blue slowly in light.
2	Limonene.	$C_{10}H_{16}$	"	"	1	"
3	Thynol.	$C_{10}H_{14}O$	Cymbenzene.	Phenol.	1	Brown.
4	Cavacrol.	$C_{10}H_{14}O$	"	"	100	Red-orange.
5	Menthol.	$C_{10}H_{18}OH$	Menthane.	Secondary alcohol.	1	Purple in light.
6	Borneol.	$C_{10}H_{17}OH$	Camphane.	"	5	Blue "
7	Terpineol.	$C_{10}H_{17}OH$	Hydroterpene.	Tertiary alcohol.	10	Purple to dirty blue.
8	Camphor.	$C_{10}H_{16}O$	Camphane.	Ketone.	10	Pink.
9	Thujone.	$C_{10}H_{16}O$	Dehydroterpene.	"	1	Dirty blue in light.
10	Pulegone.	$C_{10}H_{16}O$	"	"	1	Red-orange.
11	Cineol.	$C_{10}H_{18}O$	Menthane.	Ether.	1	Purple to gray-blue.
12	Anethol.	$C_{10}H_{12}O$	Allylbenzene.	"	1	Violet.
13	Eugenol.	$C_{10}H_{12}O_2$	"	Phenol-ether.	10	"
14	Gualacol.	$C_7H_8O_2$	Benzene.	"	2	Green.
15	Linalool.	$C_{10}H_{17}O_4$	Aliphatic hydro- terpene.	Tertiary alcohol.	10	Red-brown.

\* Concentration: cc. or gm. per 100 cc. of  $CHCl_3$  solution.

give colors which in any way resemble the color formed with cholesterol. They are pinene, limonene, and borneol. Pinene and limonene are hydrocarbons and borneol is a secondary alcohol. Since cholesterol contains a secondary alcohol group, it is not strange that a terpene with a similar group attached should give the same reaction. Menthol is also a secondary alcohol, but it will not develop the final blue. The reaction stops at the purple stage in this case.

In all of the formulæ hitherto proposed for cholesterol, the secondary alcohol group has always been attached to a menthane ring. If one were to place this secondary alcohol group on a camphane instead of a menthane ring, it would still represent all of the known reactions of cholesterol. There has been no evidence hitherto advanced which would indicate the structure of the terpene nucleus of cholesterol. The formula of Windaus<sup>16</sup> merely attempts to give some idea of a possible arrangement of the carbon atoms. This formula of Windaus could easily be modified so as to contain a camphane group on which to attach the secondary alcohol group without changing the parts of the formula determined experimentally.

Although the similarity of the reaction of cholesterol and borneol with the  $\text{SbCl}_5$  reagent may not be a sufficient basis on which to build a structural formula, yet the following modification of Windaus' latest formula for cholesterol is suggested.



<sup>16</sup> Windaus, A., *Nachr. k. Ges. Göttingen*, 1919, 237-254.

This formula merely expresses, in addition to the reactions already correlated by the formula, the similarity between cholesterol and borneol so far as the color reaction with the  $\text{SbCl}_5$  reagent is concerned. Further study of the terpenes with respect to this color reaction might possibly yield valuable data upon which to base a structural formula for cholesterol and other compounds containing terpene nuclei of unknown structure.

The action of the  $\text{SbCl}_5$  reagent upon some compounds of unknown structure, but which resemble cholesterol more nearly in probable composition, was also tested. The following are the data obtained with these sesquiterpenes, polyterpenes, and derivatives.

TABLE XXXIX.  
*Abietic Acid*\*:  $\text{C}_{20}\text{H}_{30}\text{O}_2$ . M. P.  $139^\circ$ .

Experiment No.	Concentration per 100 cc. $\text{CHCl}_3$ solution.	Amount.	Dilute with $\text{CHCl}_3$ .	$\text{SbCl}_5$ reagent.	Solution in $\text{CHCl}_3$ .	Color.	Final color in light.
	gm.	cc.	cc.	cc.	cc.		
1	1	0.1	0.5	0.2	5	Light blue.	Deeper blue.
2	1	0.2	0.5	0.2	10	" "	" "
3	1	0.5	0.0	0.2	10	Violet.	Purple to blue.

\* The abietic acid and the abietates used were exceptionally pure. They were prepared by Dr. E. O. Ellingson under Professor Kahlenberg's direction.

TABLE XL.  
*Cedrol*:  $\text{C}_{18}\text{H}_{28}\text{OH}$ . M. P.  $74^\circ$ .

Experiment No.	Concentration per 100 cc. $\text{CHCl}_3$ solution.	Amount.	Dilute with $\text{CHCl}_3$ .	$\text{SbCl}_5$ reagent.	Solution in $\text{CHCl}_3$ .	Color.	Final color in light.
	gm.	cc.	cc.	cc.	cc.		
1	1	0.5	0.0	0.2	10	Red to magenta.	Purple.
2	1	0.2	0.5	0.2	10	" " "	"

Abietic acid acts similarly to cholesterol, but the solution must be more concentrated. The blue color is developed with abietic acid almost as fast as with cholesterol.

*Zinc Abietate*.—A turquoise, quickly changing to a blue, is formed by the action of the  $\text{SbCl}_5$  reagent with a chloroform solution of zinc abietate.

*Iron Abietate*.—Iron abietate is insoluble in chloroform and therefore gave no test.

*Lead Resinate*.—A good blue was obtained with this compound.

*Colophony*.—A blue color was produced which faded more rapidly than the cholesterol blue on standing.

Wallach<sup>17</sup> has obtained pinene from rosin by destructive distillation under greatly diminished pressure. E. Kremers<sup>18</sup> suggests that rosin and abietic acid are each probably polymerization products of two molecules of pinene.

Cedrol is a tertiary alcohol and acts similarly to terpineol.

*Caoutchouc*:  $(\text{C}_5\text{H}_8)_x$ .—With the  $\text{SbCl}_5$  reagent, rubber latex forms a violet-brown precipitate which does not entirely dissolve in chloroform, but leaves a black sponge undissolved. The solution is a purplish blue changing to a slightly cloudy blue on exposure to light. Vulcanized rubber behaves similarly.

*Isoprene*:  $\text{C}_5\text{H}_8$ .—A dilute solution of isoprene in chloroform when treated with the  $\text{SbCl}_5$  reagent forms a violet solution which quickly changes to a deep blue on exposure to light. Pure isoprene treated with pure antimony pentachloride polymerizes forming a sticky rubber-like mass which also exhibits this color change.

It would seem probable that the color is produced by the combination of antimony pentachloride with a polymer of isoprene, rather than with the simple compound.

It has been noted by many investigators that the polyterpenes, like abietic acid, react similarly to cholesterol.

The colors produced by the terpenes and allied compounds with the  $\text{SbCl}_5$  reagent in chloroform are probably due to the formation of addition compounds of the terpenes with antimony pentachloride, similar to the one formed with cholesterol. It would obviously lead too far to make an attempt to isolate and analyze each one of all of the various colored compounds which are formed when antimony pentachloride reacts with the individual terpenes. Nevertheless, it was deemed to be of sufficient importance to actually isolate, purify, and analyze two typical ones; namely, those formed with pinene and with borneol respectively.

<sup>17</sup> Wallach, O., and Walker, W., *Ann. Chem. Pharm.*, 1893, cclxxi, 308; abstracted in *J. Chem. Soc.*, 1893, lxiv, 101.

<sup>18</sup> Mead, J. L., and Kremers, E., *Proc. Am. Pharm. Assn.*, 1893, xli, 209.



The methods of preparation, purification, and analysis used for the compounds of antimony pentachloride with pinene, and of antimony pentachloride with borneol, were the same as those employed in the study of the corresponding compounds with cholesterol.

The data for the analysis of the pinene compound are as follows:

*Analysis for Antimony.*

- Experiment 1.* Weight of compound taken = 0.1263 gm.  
                   "   "  $\text{Sb}_2\text{S}_3$  formed = 0.0977 " = 0.0349 gm. Sb.  
                   Amount of Sb in sample = 27.63 per cent.  
*Experiment 2.* Weight of compound taken = 0.1196 gm.  
                   "   "  $\text{Sb}_2\text{S}_3$  formed = 0.0909 " = 0.0325 gm. Sb.  
                   Amount of Sb in sample = 27.19 per cent.  
 Average of the two analyses = 27.41 per cent Sb.

*Analysis for Chlorine.*

- Experiment 1.* Weight of compound taken = 0.1250 gm.  
                   "   " AgCl formed = 0.2075 " = 0.0513 gm. Cl.  
                   Amount of Cl in sample = 41.04 per cent.  
*Experiment 2.* Weight of compound taken = 0.1361 gm.  
                   "   " AgCl formed = 0.2257 gm. = 0.0558 gm. Cl.  
                   Amount of Cl in sample = 41.00 per cent.  
 Average of the two analyses = 41.02 per cent Cl.

	Found.	Computed for $\text{C}_{20}\text{H}_{30}\text{SbCl}_5$
	per cent	per cent
Sb,	27.41	27.73
Cl,	41.02	40.90

The analytical data for the borneol compound are:

*Analysis for Antimony.*

- Experiment 1.* Weight of compound taken = 0.1322 gm.  
                   "   "  $\text{Sb}_2\text{S}_3$  formed = 0.0982 " = 0.0351 gm. Sb.  
                   Amount of Sb in sample = 26.55 per cent.  
*Experiment 2.* Weight of compound taken = 0.1310 gm.  
                   "   "  $\text{Sb}_2\text{S}_3$  formed = 0.0966 " = 0.0345 gm. Sb.  
                   Amount of Sb in sample = 26.34 per cent.  
 Average of the two analyses = 26.45 per cent Sb.

*Analysis for Chlorine.*

- Experiment 1.* Weight of compound taken = 0.1208 gm.  
                   "   " AgCl formed = 0.1909 " = 0.0472 gm. Cl.  
                   Amount of Cl in sample = 39.07 per cent.

*Experiment 2.* Weight of compound taken = 0.1951 gm.

" " AgCl formed = 0.3102 " = 0.0767 gm. Cl.

Amount of Cl in sample = 39.31 per cent.

Average of the two analyses = 39.19 per cent Cl.

	Found.	Computed for $C_{27}H_{46}O \cdot SbCl_5$
	per cent	per cent
Sb,	26.45	26.62
Cl,	39.19	39.27

It is evident from the above analyses that the compounds formed by antimony pentachloride with pinene and borneol, respectively, are analogous to the compound formed with cholesterol. Therefore, one molecule of pinene combines with one molecule of antimony pentachloride, and one molecule of borneol combines with one molecule of antimony pentachloride. It will be recalled that in the case of cholesterol, the compound was equally simple in character; namely, one molecule of cholesterol to one molecule of antimony pentachloride. The similarity between the reactions of antimony pentachloride and cholesterol on the one hand, and of antimony pentachloride and the various terpenes on the other hand, would argue in favor of the view that cholesterol is really a terpene-like compound.

#### SUMMARY.

1. With a dilute solution of cholesterol, a chloroform solution of  $SbCl_5$  forms a muddy brown precipitate which upon solution in more chloroform yields a clear, purple liquid which quickly changes to a cobalt-blue upon exposure to light.

2. The blue color is fairly stable upon heating, but upon long exposure to light it slowly fades and changes to a greenish hue. The blue color is very stable at low temperatures in the dark.

3. The compound which forms the blue coloration in chloroform may be obtained in pure form by first preparing the muddy brown precipitate with cholesterol and antimony pentachloride, and then, by means of carbon tetrachloride, washing this free from the excess of both cholesterol and antimony pentachloride.

4. The pure compound upon analysis for antimony, chlorine, and carbon yields results which lead to the conclusion that it is a simple addition product of the formula  $C_{27}H_{46}O \cdot SbCl_5$ . The molecular weight by the boiling point method in ether was found to confirm this conclusion.

5. A slight modification of the Henz method for the determination of antimony as trisulfide has been developed; namely, the sulfide is precipitated with hydrogen sulfide from a chloroform solution.

6. Since the blue compound is quite definite in composition, the color reaction between cholesterol and antimony pentachloride is perfectly quantitative and may be used successfully in the colorimetric estimation of cholesterol.

7. Favorable results have been obtained by using this colorimetric method for the analysis of the cholesterol content of blood.

8. Phytosterol undergoes a similar reaction with the  $\text{SbCl}_5$  reagent except that the blue color is not developed as rapidly as with cholesterol. The reaction is also quantitative in the case of phytosterol.

9. These color reactions afford a means of testing the comparative purity of different samples of cholesterol and phytosterol.

10. Chlorides, other than antimony pentachloride, were found to yield no similar colored compounds.

11. The brown precipitate formed with the  $\text{SbCl}_5$  reagent and cholesterol is very sparingly soluble in carbon tetrachloride, yielding a salmon-pink solution. In ethyl alcohol it forms a brilliant green color. Neither of these solvents, however, has any advantage over chloroform for colorimetric determinations. The brown precipitate can be formed only in chloroform and carbon tetrachloride, but it is soluble in various other solvents yielding different colored solutions.

12. The vegetable oils, due to their high content of unsaturated fatty acids, form a brown color with the  $\text{SbCl}_5$  reagent. Animal oils high in cholesterol and low in the unsaturated fatty acids, show the blue cholesterol reaction. Some of the mineral oils exhibit a slight tendency toward a blue coloration with the  $\text{SbCl}_5$  reagent. This color reaction may be used to differentiate between hydrogenated and unhydrogenated oils.

13. Isocholesterol and esters of cholesterol produce the same blue color. The lipoids do not. The essential oils produce various colored solutions with the  $\text{SbCl}_5$  reagent.

14. Many of the terpenes and camphors react with the  $\text{SbCl}_5$  reagent forming colored solutions.

15. On the basis of the reaction of the  $\text{SbCl}_5$  reagent with borneol a modified structural formula for cholesterol has been suggested.

16. Abietic acid, colophony, and caoutchouc all produce blue colors with the  $\text{SbCl}_5$  reagent. Isoprene also forms a blue solution, but this blue may be due to a polymer of isoprene, formed by the action of the antimony pentachloride on isoprene.

17. Addition compounds of antimony pentachloride with pinene and borneol, respectively, were prepared, purified, and analyzed. Their composition is represented by the formulas  $\text{C}_{10}\text{H}_{16} \cdot \text{SbCl}_5$  and  $\text{C}_{10}\text{H}_{17}\text{OH} \cdot \text{SbCl}_5$ , respectively.



# COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

## VIII. ACETYLATION OF AMINO COMPOUNDS.

By JOSEPH B. MUENZEN, LEOPOLD R. CERECEDO, AND  
CARL P. SHERWIN.

(From the Chemical Research Laboratory of Fordham University, New York.)

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In the literature are found many contradictory statements regarding the fate of the amino benzoic acids in the animal organism. According to Salkowski (1) *m*-amino benzoic acid is detoxicated in man, the dog, and the rabbit chiefly by a conversion into the corresponding uramino benzoic acid, though small amounts of it are joined through the carboxyl group with glycocoll to form amino hippuric acid. According to Fränkel (2) *o*-amino benzoic acid is excreted unchanged, while the methyl ester of *o*-acetyl amino benzoic acid is hydrolyzed in the animal body, resulting in the excretion in the urine of the methyl ester of anthranilic acid. According to Hensel (3), also Ellinger and Hensel (4), the *m*- and *p*-amino benzoic acids are acetylated in the body of the rabbit and excreted as the respective acetyl amino benzoic acids. Lastly, Hildebrandt (5) asserts that all three of the amino benzoic acids are excreted unchanged by the dog.

We recently found (6) that *p*-amino phenylacetic acid when fed to human beings or rabbits was acetylated, but when fed to a dog only *p*-amino phenaceturic acid (*p*-amino phenacetyl glycocoll) appeared in the urine. In none of these cases, however, did we find a uramino compound, as reported by Salkowski for the benzoic acid homologue, nor did we find a mixture of two different types of detoxication products in the urine of the same subject as he claims to have done. We also fed to a man, as well as to dogs and to rabbits, *o*-, *m*-, and *p*-amino benzoic acids, and found that the *o*-amino benzoic acid was excreted free in the urine of all of these subjects; the *m*-amino benzoic acid was

excreted free by the dog, but by rabbits and by man it was eliminated as *m*-acetylamino benzoic acid; while the *p*-amino benzoic acid was acetylated to even a larger extent than the *m*-amino benzoic acid by both rabbits and man, being largely excreted as *p*-acetylamino benzoic acid. In no case did we find a uramino compound or a hippuric acid as reported by Salkowski. Moreover, though we did find that *o*-amino benzoic acid was excreted unacetylated by the different subjects, still it seemed strange that the *o*-acetylamino benzoic acid should be hydrolyzed into acetic and benzoic acids as Fränkel reports (2). We, therefore, fed *o*-acetylamino benzoic acid to rabbits and dogs and found that it was less toxic than the anthranilic acid (*o*-amino benzoic acid) and was excreted in the same form in which it was fed, that is, as the acetylated acid. Lastly, *p*-hydrazino benzoic acid was fed to a dog to determine its course of metabolism. Contrary to statements in the recent literature (7) we found it to be so toxic that it could not be given *per os*, and even after the subcutaneous injection of only 0.5 gm. into a large dog death followed in about 6 hours. Only a trace of the original acid was recovered from the urine, and this unchanged.

Lest our failure to find *p*-amino hippuric acid in the urine after feeding *p*-amino benzoic acid might have been due to ignorance of the constants, we synthesized the *p*-amino hippuric acid, which was as yet unknown, and studied it.

#### EXPERIMENTAL.

##### *o*-Amino Benzoic Acid.

A dog of 12.5 kilos body weight was fed 4 gm. of *o*-amino benzoic acid in the form of a solution of the sodium salt. The following day a second dose of 4 gm. was given. The dog showed no evidence at all of intoxication, although the substance has been stated to be toxic, but ate well throughout the course of the experiment. Nor were there any signs of hemoglobinuria. The urine was then collected until 48 hours after the second feeding. It did not reduce Fehling's solution nor show a positive test with Millon's reagent, evidencing the absence both of a glycuronic acid conjugate and of a phenolic substance resulting from an oxidation in the ring. The urine was then evaporated to a thick

syrup on the water bath, cooled, acidified to Congo red with phosphoric acid, and extracted in a rotary extractor for a period of 12 hours with ethyl acetate. The ethyl acetate was then evaporated almost to dryness, taken up with 350 cc. of hot water, and boiled with charcoal. Only uncombined *o*-amino benzoic acid was found, for after allowing the solution to evaporate gradually at room temperature for several days, crystals of *o*-amino benzoic acid separated out, and no other product could be obtained. Thus 3.8 gm. of the original material were recovered. After recrystallization from water it melted at 142–144°C.

A rabbit received 1 gm. of *o*-amino benzoic acid on each of 2 successive days. The urine was collected and treated as described above. Only free *o*-amino benzoic acid was isolated, and of this only 0.95 gm.

A man weighing 61 kilos ingested 4 gm. of *o*-amino benzoic acid during the course of 4 days. The only substance that could be found in the urine was the uncombined acid, and only about 1 gm. of this.

#### *o*-Acetylamino Benzoic Acid.

A rabbit was fed 1 gm. of *o*-acetylamino benzoic acid on each of 3 successive days. The urine, treated as described above, yielded 2.2 gm. of the unchanged substance, which after two recrystallizations from water melted within 1 to 2 degrees of the melting point of *o*-acetylamino benzoic acid.

Another rabbit was fed 3 gm. of *o*-acetylamino benzoic acid. This time also more than 2 gm. were recovered unchanged, nor was there any indication of a cleavage of the acetyl radical from the amino group.

A dog received 8 gm. of *o*-acetylamino benzoic acid during the course of 2 days. From the urine 6 gm. of the unchanged substance were recovered.

#### *m*-Amino Benzoic Acid.

A dog was given 4 gm. of *m*-amino benzoic acid on each of 3 consecutive days. The urine was treated as usual and extracted repeatedly with ethyl acetate. The ethyl acetate was then evaporated and the united residues taken up in water and purified.



Between 6 and 7 gm. of *m*-amino benzoic acid were thus recovered. The urinary residue was then strongly acidified again with phosphoric acid and extracted with various other organic solvents. It was also distilled with steam. Finally, in order to split any conjugation product, a portion of the urine was heated under a reflux with 25 per cent phosphoric acid for a period of 6 hours and then distilled again with steam. We were unable, however, to trace any more of the *m*-amino benzoic acid by these extra measures.

In order to check up the accuracy of our method, therefore, we collected a 48 hour portion of urine from the same dog, made it slightly alkaline with sodium carbonate, and added to it 5 gm. of *m*-amino benzoic acid. We then analyzed the urine as before. Of the 5 gm. of *m*-amino benzoic acid added to the urine 3.48 gm. (70 per cent) were thus recovered.

Each of three rabbits was fed 3 gm. of *m*-amino benzoic acid at the rate of 1 gm. per day. From the urine of the first rabbit we obtained 1.76 gm. of *m*-acetylamino benzoic acid; from that of the second, 1.23 gm.; and from that of the third, 1.19 gm. Thus from the three rabbits, after feeding 9 gm. of the substance, 4.18 gm. of the acetylated product were isolated. A small amount of the material was also recovered unchanged.

As the *m*-amino benzoic acid proved to be non-toxic to dogs and rabbits, each of two men easily tolerated 5 gm. of the substance. This was the dose formerly taken by Salkowski. Though an elaborate method was followed for the identification of a uramino compound, we were much surprised to find only the acetylated product. This was easily and effectually extracted from the urine with ethyl acetate following the technique previously employed in the cases of the dog and rabbit. Though the acetylation was incomplete, just as it was in the case of the rabbit, still no detoxication product other than the acetyl compound could be found. A very small amount of the original uncombined acid, however, was also recovered. From the urine of one man 1.19 gm. of the acetylated amino benzoic acid was isolated, and from that of the other, 1.37 gm. After recrystallization, this melted at 246°C. The synthetic compound, prepared by the action of acetic anhydride on *m*-amino benzoic acid, melted at 248–249°C.

Analysis of the compound for nitrogen according to Kjeldahl gave the following results.

Substance taken.	0.1 N acid required.	Nitrogen found.	Nitrogen calculated.
gm.	cc.	per cent	per cent
0.1677	9.25	7.72	7.82
0.2004	11.00	7.68	7.82

*p*-Amino Benzoic Acid.

A dog was fed 8 gm. of *p*-amino benzoic acid. From the urine only 3.8 gm. of the free acid were recovered, but no conjugation product of any kind could be found.

Two rabbits received 3 gm. each of the *p*-amino benzoic acid during the course of 3 days. From the urine of one we obtained 1.96 gm. of *p*-acetylamino benzoic acid, and from that of the other, 2.16 gm.

Each of two men ingested 5 gm. of *p*-amino benzoic acid at a single dose. From the urine of the first we isolated 3.48 gm. of *p*-acetylamino benzoic acid, and from that of the second, 2.72 gm. This material, after two recrystallizations from hot water, melted at 247–249°C. Kjeldahl nitrogen determinations yielded the following results.

Substance taken.	0.1 N acid required.	Nitrogen found.	Nitrogen calculated.
gm.	cc.	per cent	per cent
0.2642	14.75	7.81	7.82
0.1743	9.60	7.71	7.82

*Synthesis of p*-Amino Hippuric Acid.

This compound was prepared by the reduction of *p*-nitro hippuric acid, which substance was prepared in the usual way by the Schotten-Baumann reaction from glycocholl and nitrobenzoyl chloride. The substance melted at 131–132°C. while Jaffé gives 129°C.

*p*-Nitro hippuric acid, 8 gm., was dissolved in ammonium sulfide solution. Hydrogen sulfide was then passed into this solution for 4 hours. The solution was evaporated on the water

bath three times, to remove completely the ammonium sulfide. The precipitated sulfur was then filtered off, the filtrate concentrated to a small volume, and acidified with dilute hydrochloric acid. A light brown substance separated out. This was filtered off and recrystallized from hot water. After being carefully dried in a desiccator over concentrated sulfuric acid it melted at 199°C. Yield, 3.0 gm. The substance is soluble in alcohol, benzene, chloroform, acetone, and hot water; it is insoluble in cold water, ether, and carbon tetrachloride. It crystallizes from hot water in short, irregular prisms. It gave the following analysis.

	C	H	N
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Found .....	55.78	5.10	14.30
	55.73	5.37	14.38
Calculated for $C_8H_{10}N_2O_4$ .....	55.67	5.20	14.43

*p*-Hydrazino Benzoic Acid.

A dog of 12 kilos body weight received 1 gm. of this substance *via stomach tube* as a water solution of the sodium salt. After about half an hour the animal showed marked signs of intoxication and was seized with a violent fit of vomiting. A week later, after the dog had recovered, a dose of 0.5 gm. of *p*-hydrazino benzoic acid was injected subcutaneously as a water solution of the sodium salt. Within 45 minutes the dog showed much the same symptoms as before. He was seized with violent fits of spasmodic vomiting. Following this there was a drop of more than 1 degree in body temperature, a slowing of the circulation, and a gradual decrease in the respiratory rate. At the end of 6 hours the dog died, apparently from a gradual paralysis of the respiratory muscles.

Only the merest traces of the unchanged substance could be detected in the urine. Apparently the material is so toxic that there is no attempt on the part of the liver or tissues as a whole at detoxication. The symptoms here described are not specifically those of *p*-hydrazino benzoic acid intoxication but are rather the ordinary symptoms of hydrazine poisoning.

## DISCUSSION.

Up to the present few cases of acetylation have been reported and, as far as we can ascertain, no reaction of this kind has been noted in connection with work on normal human subjects. The first case reported was that of Baumann and Preusse (8) who isolated an acetylated cysteine conjugate of bromobenzene after feeding bromobenzene to dogs. It seems peculiar that this reaction cannot be reproduced in other animals. Dogs, cats, rabbits, pigs, and human beings (9) all excrete a large amount of ethereal sulfates after bromobenzene ingestion, but no mercapturic acid. Cohn (10) later showed that rabbits, after receiving *m*- and *p*-nitro benzaldehydes, excrete the corresponding acetylated benzoic acids in their urine, but that dogs fail to reduce the nitro groups of these substances and simply excrete the compounds as the corresponding hippuric acids. Knoop and Kertess (11) fed *dl*- $\gamma$ -phenyl- $\alpha$ -amino butyric acid to dogs, and isolated from the urine only *d*- $\gamma$ -phenyl- $\alpha$ -acetylamino butyric acid. Dakin (12) fed *dl*-*p*-methyl phenylalanine to a subject with alcaptonuria and found a small amount of *d*-acetylamino-*p*-methyl phenylalanine in the urine. Normal individuals apparently oxidize this substance completely.

Recently it has been found that fowls (13), like rabbits and human beings, will acetylate *m*-amino benzoic acid. Rabbits also acetylate *o*-amino phenylacetic acid (14) and excrete it as acetyldioxyindole. Human beings, however, and dogs fail to acetylate this substance.

From the data available there seems to be a sharp division in the various species as to their ability to employ the acetylation reaction as a defense against foreign amino compounds. The dog quite readily acetylates *aliphatic* amino compounds or *aromatic side chain* amino compounds; rabbits and human beings, however, fail to acetylate these types of compounds, but make use of the reaction when the amino group is attached to the *ring*.

## SUMMARY.

*o*-Amino benzoic acid (anthranilic acid) is excreted unchanged in the urine of men, dogs, and rabbits. Acetyl-*o*-amino benzoic acid is excreted unchanged in the urine of dogs and rabbits and is not hydrolyzed.

*m*-Amino benzoic acid is excreted unchanged in the urine of dogs, but is acetylated by the human organism as well as by rabbits, contrary to Salkowski who reports the finding of a uramino benzoic acid and also some *m*-amino hippuric acid in each case after feeding *m*-amino benzoic acid.

*p*-Amino benzoic acid is excreted free after ingestion by dogs, but is acetylated by rabbits and human subjects. This is believed to be the first case reported where acetylation was employed by a normal human subject as a detoxication reaction.

*p*-Hydrazino benzoic acid was fed and injected. It was found to be so toxic that an injection of 0.5 gm. killed a dog weighing 12 kilos in less than 8 hours. Death was caused by a progressive slowing of the pulse rate and gradual paralysis of the respiratory muscles—the symptoms of hydrazine poisoning.

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# THE STUDY OF BENZOIC ACID CONJUGATION IN THE DOG WITH A DIRECT QUANTITATIVE METHOD FOR HIPPURIC ACID.

By ARMAND J. QUICK.

*(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)*

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Extensive research on the fate of ingested benzoic acid has led to the general conclusion that sublethal doses are eliminated almost quantitatively as hippuric acid by all animals studied with the notable exception of the dog. Scant attention has been paid to conjugated compounds of benzoic acid other than hippuric acid although Salkowski (1) as early as 1877 observed the presence of a reducing substance in the urine of rabbits, dogs, and man when benzoic acid had been fed. Kobert (2) called the reducing substance glycuronic acid, but Siebert (3) was the first definitely to identify it in the urine of animals. He failed to realize, however, that the glycuronic acid was conjugated with benzoic acid, and it remained for Magnus-Levy (4) to demonstrate that benzoic acid when fed to a sheep could and did combine with glycuronic acid and was eliminated as such through the kidney. This occurred, however, only when large doses of benzoic acid were fed, indicating perhaps that this type of conjugation is a second line of defense which is called into play when the available supply of glycine is temporarily exhausted. The human organism apparently behaves like that of the sheep since Dakin (5) could detect a reducing substance in the urine of men who had taken relatively large doses of benzoic acid. Recently Csonka (6) in studying the elimination of benzoic acid in the pig found that the percentage conjugated with glycuronic acid varied from 9.7 to 45.4 per cent depending on the amount of glycine in the diet, thus indicating that in this animal the glycuronic acid conjugation can assume an importance approaching that of the hippuric acid synthesis.

It is rather striking that the dog has been seldom used in hippuric acid studies and that the few researches reported have not yielded definite and concordant results. Wiener (7) stated that the dog was unsuited because the hippuric acid formed was partly hydrolyzed by the enzyme, histozyme, thus giving rise to free benzoic acid in the urine. Brugsch and Hirsch (8) who were among the first investigators to study the hippuric acid synthesis in the dog stated that they could recover only 40 to 75 per cent of the benzoic acid fed and that only about 5 to 15 per cent was bound. They recorded that the urine reduced Fehling's solution and had a small positive rotation. Since no conjugated glycuronic acids having a positive rotation were known at that time, and since acid hydrolysis did not increase the positive rotation, the authors concluded that the reducing substance was not glycuronic acid. Magnus-Levy (4) using their data to calculate benzoyl glycuronic acid also concluded that little glycuronic acid was formed by the dog. Rost, Franz, and Weitzel (9) after extensive studies concluded that not more than one-half to two-thirds of the benzoic acid fed was excreted as hippuric acid. While they did observe the presence of a reducing substance, which they assumed was glycuronic acid, following the ingestion of large doses of benzoic acid, the analytical methods at their disposal were too inadequate to enable them to differentiate between the various forms of benzoic acid. In their determination of hippuric acid, they followed the general procedure outlined by Bunge and Schmiedeberg (10), which as Folin and Flanders (11) have stated, is neither accurate nor convenient. One of the greatest errors in the method and of its many modifications is due to the evaporation of the urine which has been made alkaline, for not only is a portion of the hippuric acid split, as Folin and Flanders have demonstrated, but benzoyl glycuronic acid is hydrolyzed to an even greater degree, as will be shown later. Undoubtedly most of the free benzoic acid which Rost and the other workers found resulted from benzoyl glycuronic acid by hydrolysis due to the procedure employed.

In order to study the conjugation of benzoic acid in an animal like the dog which excretes both conjugated forms of benzoic acid, it is very desirable to have direct analytical methods for

hippuric acid and benzoyl glycuronic acid. The methods of Folin and Flanders (11) and of Kingsbury and Swanson (12), although designated as procedures for hippuric acid, determine really total benzoic acid. The error produced by calculating total benzoic acid as hippuric acid when other forms of conjugated benzoic acid are present will be apparent. Csonka (6) met this difficulty by determining free and total benzoic acid and glycuronic acid, from which data he calculated hippuric acid by difference. Unfortunately, no method is known at present whereby benzoyl glycuronic acid can be separated from the other reducing substances either normally or pathologically present in the urine.

Snapper and Laqueur (13) have proposed a method which might be considered a more direct determination for hippuric acid. The urine is extracted with several portions of ethyl acetate, and the extract, after the solvent has been removed by distillation, is treated with alkaline hypobromite solution to destroy the urea carried over by the solvent. The residue is analyzed by the Kjeldahl method for nitrogen from which hippuric acid can be calculated. The removal of urea is not complete, however, and the empirical correction which has to be made for the undecomposed portion is a serious disadvantage inherent in the method.

Since the glycyl radical is the characteristic portion of the hippuric acid molecule it appeared that any satisfactory method for glycine might also serve as a direct means for determining hippuric acid. Although such a method has been outlined by Henriques and Sørensen (14), it has received scant attention. In their method the hippuric acid, which is removed from urine by repeated extractions with ethyl acetate, is hydrolyzed with strong hydrochloric acid, and the liberated glycine determined by the well known formol titration. Van Slyke (15) has suggested his gasometric method for amino nitrogen as an alternative means to analyze for glycine. As already mentioned, Snapper and Laqueur (13) have recently found that ethyl acetate also extracts appreciable amounts of urea, and since this on hydrolysis yields ammonia which reacts in both methods, an error of considerable importance is introduced.

Since the determination of glycine seemed to offer the best quantitative method for hippuric acid, the most favorable con-



ditions for the isolation of the acid from urine and for its hydrolysis were studied. It was soon found that ether is superior to ethyl acetate since it extracts all the hippuric acid and practically no urea, and it is, furthermore, better adapted for a continuous extraction apparatus, the use of which eliminates a great amount of tedious mechanical work.

Since no satisfactory way could be found for removing benzoyl glycuronic acid quantitatively from urine, its direct determination by a sugar method was investigated. The lead acetate precipitation, which Csonka employed, was omitted since it effects no satisfactory separation of glycuronic acid from the other reducing constituents of the urine. It was found that the glycuronic acid could be determined directly in urine by the Shaffer-Hartmann method for glucose (16).

With methods available for the determination of benzoic acid in the various forms that it might occur in the urine of the dog, the study of the conjugation of the acid in that animal was undertaken. In this paper are reported the results which were obtained when the animal was kept on rations low and high in glycine.

#### *Analytical Methods.*

*Determination of Hippuric Acid.*—A 20 cc. sample of urine was taken unless the hippuric acid content was known to be high, when a proportionately smaller sample was used. After acidification with 0.5 cc. of 5.0 N sulfuric acid, the urine was extracted for 3 hours with ether in a continuous extractor. The Erlenmeyer flask containing the ether extract was then connected with a condenser and the ether distilled off. The residue which was usually crystalline was dissolved in 10 cc. of concentrated hydrochloric acid and refluxed on a hot plate under an air condenser for 30 minutes. On cooling, the solution was carefully transferred to a small glass dish and evaporated to dryness on a water bath. In this process most of the benzoic acid volatilized. The residue was dissolved in a small amount of hot water and, if the resulting solution was too deeply colored, it was treated with a little decolorizing charcoal (norit). Since charcoal was found sometimes to contain a small amount of ammonia, a blank was run. Decolorization was rarely found necessary unless the urine contained much

benzoyl glycuronic acid, a small amount of which would be extracted and on hydrolysis yield colored decomposition products. The solution was filtered into a 25 cc. volumetric flask, a drop of a 1 per cent neutral red indicator solution added, and the solution nearly neutralized with  $N$  sodium hydroxide. The final adjusting of the solution to pH 7 was carried out by adding 0.1  $N$  sodium hydroxide until the resulting color matched the prepared standard. The flask was now filled to the mark with recently boiled distilled water, mixed, and a 20 cc. aliquot transferred to a 100 cc. Erlenmeyer flask. To this 6 drops of 1 per cent phenolphthalein and 10 cc. of neutralized formaldehyde (40 per cent) were added, and the solution titrated with 0.1  $N$  sodium hydroxide until the deep red color matched the standard which was prepared by mixing 20 cc. of neutral distilled water, 10 cc. of neutralized formaldehyde, 6 drops of phenolphthalein, 2 drops of neutral red, and 0.3 cc. of 0.1  $N$  sodium hydroxide. Although the solutions analyzed were often somewhat colored no trouble was encountered in securing a definite end-point, the extra drop of neutral red in the standard permitting a better match between the two solutions.

Since 1 cc. of 0.1  $N$  sodium hydroxide is equivalent to 1 cc. of 0.1  $N$  glycine, the titration value, corrected for the 0.3 cc. blank, can be converted directly either to hippuric or benzoic acid.

As an alternative to the formol titration, Van Slyke's gasometric method for amino nitrogen was used. 1 or 2 cc. of the neutralized solution remaining after the aliquot for the formol titration had been removed were analyzed. The volume of gas obtained was multiplied by 0.93, the factor to correct for the abnormal volume of gas given off by glycine.

The method as outlined when tested on pure solutions of known concentrations of hippuric acid yielded recoveries that were over 95 per cent of the theoretical. The following data were obtained on a specimen of human urine voided 2 hours after the ingestion of 2 gm. of sodium benzoate.

Volume of urine.....	200 cc.
Total benzoic acid excreted.....	1.47 gm.
Benzoic acid combined as hippuric acid:	
Sample I. 20 cc. Time of extraction, 3 hours. Benzoic acid by formol titration method.....	1.44 gm.

Sample II. 10 cc. Time of extraction, 2 hours.

Benzoic acid by formol titration method..... 1.41 gm.

Sample III. 20 cc. Time of extractions, 3 hours.

Benzoic acid by formol titration method..... 1.41 gm.

“ “ “ Van Slyke's method..... 1.44 “

The extractor employed was similar to the one described for the determination of menthol glycuronic acid (17) except that the Kjeldahl flask was replaced by a glass tube of sufficient internal diameter to accommodate the extraction tube, and with a constricted stem at one end to fit a 100 cc. Erlenmeyer flask. An extraction tube with an internal diameter of 15 mm. and a length of 290 mm. is well adapted for either 20 or 25 cc. samples. The funnel tube should be 355 mm. long and 7 mm. in diameter. The extraction tube is suspended in the jacket by means of a small copper wire.

*Benzoyl Glycuronic Acid.*—5 cc. of urine neutralized to litmus were diluted with 45 cc. of water and analyzed by the Shaffer-Hartmann sugar method. The combined carbonate citrate reagent was used, and the procedure as outlined for glucose followed exactly. The reducing power as found in terms of glucose was converted to glycuronic acid by multiplying by 1.085. This factor was determined experimentally using pure menthol glycuronic acid.

*Free Benzoic Acid.*—The method of Raiziss and Dubin (18) was employed. As the free benzoic acid was found to be small, i.e. 0.03 to 0.15 gm. in a 30 hour specimen of urine, it was only occasionally determined.

*Total Benzoic Acid.*—The method of Kingsbury and Swanson (12) was used. It was found that toluene could satisfactorily be substituted for chloroform.

#### EXPERIMENTAL.

Female dogs were used. All the animals were kept on a standard diet which was essentially that used by Karr (19).<sup>1</sup>

<sup>1</sup> Standard diet per kilo of body weight:

	gm.
Commercial casein.....	6.3
Sucrose.....	4.5
Lard.....	4.1
Bone ash.....	0.4
Salt mixture.....	0.2
Brewers' yeast.....	0.4
Water.....	2.5

They were fed once a day and when sodium benzoate was given it was always mixed with the food. It might be noted here that benzoic acid was always administered as the sodium salt. The dogs generally ate the food immediately, but occasionally forced feeding was necessary. Attempts to give sodium benzoate in solution by stomach tube proved unsatisfactory since it often caused vomiting.

The diet given was practically glycine-free. When it was desired to add glycine to the diet either the free acid or gelatin, which according to Dakin (20) contains over 25 per cent glycine, was fed.

None of the dogs was catheterized. As nearly as possible a 30 hour specimen of urine was collected since the excretion of benzoic acid was nearly complete in that period. The urine was preserved with toluene and a few cc. of dilute sulfuric acid unless an analysis for free benzoic acid was desired, when sodium fluoride and sodium dihydrogen phosphate were used instead.

#### DISCUSSION OF RESULTS.

In considering the quantitative data one finds that the sum of the combined benzoic acid is greater than the total benzoic acid determined directly. This can be accounted for by the fact that the benzoyl glycuronic acid determination is too high because it is calculated from the reducing power of the urine, and it is well known that the urine normally contains reducing substances other than glycuronic acid. With a few exceptions the calculated combined benzoic acid was about 0.2 gm. greater than total benzoic acid. Since the determination of total benzoic acid and of hippuric acid is more reliable and accurate, it is safer to calculate benzoyl glycuronic acid by difference, using, however, the direct determination as a check.

A study of Table I shows plainly the importance of the conjugation of glycuronic acid with benzoic acid in the dog. In considering the results obtained with diets containing little or no glycine, it is apparent that the amount of hippuric acid excreted is comparatively small and relatively fixed in amount, in contrast with the benzoyl glycuronic acid. This is illustrated by the first three pairs of results recorded in Chart I. In the

TABLE I.  
*The Relative Production of Hippuric Acid and Benzoyl Glycuronic Acid.*

Experiment No.	Date.	Dog.	Weight. kg.	Urine volume. cc.	$\alpha_D$	Benzoyl acid fed. gm.	Benzoic acid excreted, in urine.				Remarks.
							Total. gm.	Sum of com- bined forms. gm.	As benzoyl gly- curonic acid. gm.	As hippuric acid. gm.	
1	July 19 1905	2	8	470		3.38	3.18	3.40	2.48	0.92	72 gm. of meat fed.†
2	" 21			340		3.38	3.18	3.17	2.34	0.83	Standard diet.
3	" 23			400		3.38	3.26	3.52	2.61	0.91	"
4	" 25			250		0.85	0.88	1.09	0.50	0.59	72 gm. of meat fed.
5	" 27			320	-0.04	3.38	3.20	3.36	2.43	0.93	72 " " "
6	Sept. 29		10	270		0.19	0.43	0.37	0.37	0.09	Standard diet.
7	Oct. 2			565	-0.10	4.24	3.90	3.88	2.64	1.24	" " + 5 gm. glycine fed.
8	" 6			315	-0.07	4.24	3.35	3.53	2.62	0.91	"
9	" 10			685	-0.07	4.24	3.73	3.85	2.10	1.65	50 gm. of gelatin fed.‡
10	July 20	4	10	460		2.12	2.14	2.49	1.62	0.87	72 " " meat fed.
11	" 26			360		4.24	3.97	4.16	3.05	1.11	Standard diet.
12	" 28			720		4.24	3.69	3.97	2.39	1.58	" " + 22 gm. of gelatin fed.
13	" 22	5	7	430	0.0	4.24	3.84	4.25	3.10	1.15	72 gm. of meat fed.
14	" 24			270		2.96	2.57	3.04	1.92	1.10	72 " " " + 2.15 gm. of gly- cine fed.
15	" 26			200			3.16	3.26	0.40	2.86	Standard diet + 5 gm. of hippuric acid fed.

16	July 28	6	11	480	0 0	2.96	2.83	3.08	2.17	0.91	32.2	Standard diet.
17	Sept. 29			460	-0.25		0.34			0.18	53.0	"
18	Oct. 2			830	-0.20	4.65	4.35	4.53	2.81	1.72	39.6	"
19	" 5			570	-0.12	4.65	3.72	4.30	2.60	1.76	47.4	+ 5 gm. of glycine fed.
20	" 7			440	-0.16	4.65	3.70	3.88	2.78	1.10	29.8	60 gm. of gelatin fed.†
21	" 10			325	+0.05	4 65	4.16	4.23	2.65	1.58	38.5	Standard diet.
												60 gm. of gelatin fed.†

\* The rotation observed through a 2 dm. tube using sodium light.

† When meat was fed, it replaced part of the casein, but the total nitrogen of diet was kept constant.

‡ Gelatin replaced all casein.

## Determination of Hippuric Acid

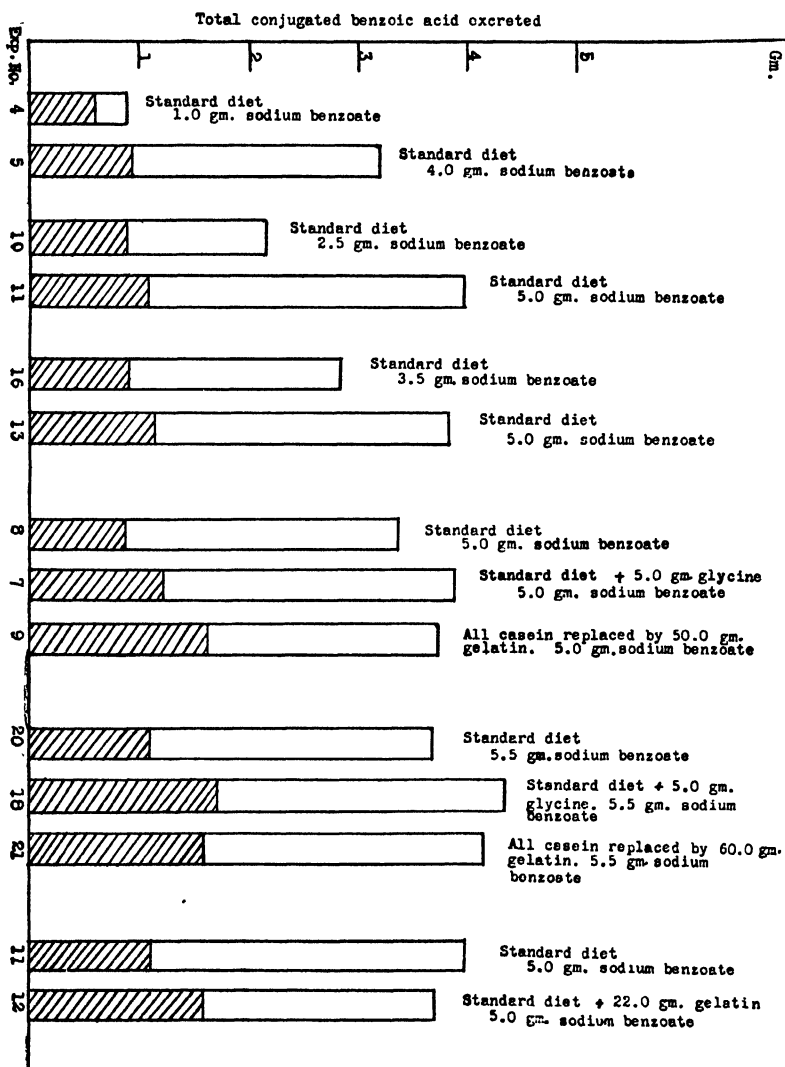


CHART I. The relative production of hippuric acid and benzoyl glycuronic acid. In the first three pairs of experiments the effect of the dosage of benzoic acid is shown, while in the other eight experiments the influence of exogenous glycine on hippuric acid synthesis is studied. The numbers below the columns refer to the experiments as listed in Table I. The shaded portion represents benzoic acid combined as hippuric; the unshaded portion, benzoic acid combined as benzoyl glycuronic acid.

first, it is found that even though the total benzoic acid excreted was increased  $3\frac{1}{2}$  times, the hippuric acid rose only 63 per cent, while the glycuronic acid increased 7 times. These results differ strikingly with those obtained on other animals. In fact, Csonka is the only investigator who, in his work on the pig, observed a similar though less marked variation. In other animals studied, on the contrary, the hippuric acid production is very elastic and takes care of all the benzoic acid unless excessive doses are fed. In the past, these variations in species were accounted for by the theory that the power of conjugating toxic substances is better developed in the herbivora than in the carnivora. If the results of hippuric acid studies on different animals are compared, however, one fails to find a clear-cut distinction between these two classes of animals. To be sure, the synthesis of hippuric acid is very limited in the dog, but, as already mentioned, this power of synthesis is also limited in the pig, which in its habits is omnivorous. Man, on the other hand, also subsists on a mixed diet, yet has the ability of excreting large quantities of benzoic acid quantitatively as hippuric acid. It might also be noted that the toxic dose of benzoic acid for dogs is approximately the same as for rabbits; namely, 1 gm. per kilo of body weight. In view of these considerations, one is justified in doubting the validity of the assumption that the power of conjugation is more highly developed in the herbivora than in the carnivora. Further study is needed before definite conclusions can be drawn, and it is especially desirable to get more information concerning detoxication mechanisms in the carnivora.

It seemed interesting to see whether the hippuric acid conjugation could be increased by feeding glycine or a protein such as gelatin which is very rich in glycine. It was found that while the presence of large amounts of either free or combined glycine did definitely raise the production of hippuric acid, the increase was comparatively small. Thus, when one dog was fed 50 gm. of gelatin, which theoretically contains enough glycine to conjugate with 20 gm. of benzoic acid, the increase of benzoic acid combined with glycine was only 0.66 gm. as compared with the output of hippuric acid on a glycine-free diet. The results obtained with free glycine were of the same order. There is no obvious explanation for these results but several possible factors may be



mentioned. The work of Bunge and Schmiedeberg (10) and the recent studies of Snapper, Grünbaum, and Neuberg (21) indicate that in the dog the kidney is the only organ that can synthesize hippuric acid, while the work of Friedmann and Tachau (22) shows that in the rabbit the liver also possesses this synthetic power. In view of these considerations, it may be that the destruction or deamination of glycine takes place so promptly that little exogenous glycine reaches the site of hippuric acid synthesis in the dog. Another explanation which should not be overlooked is the possible difference in the rate of synthesis of benzoyl glycuronic acid as compared with hippuric acid. Further work is in progress and attempts will be made to obtain results bearing on these questions.

Since the specific rotation of sodium benzoyl glycuronate according to Magnus-Levy (4) is  $[\alpha]_D^{20} = +43.83$ , it seemed remarkable that all samples of urine examined, with the exception of one which had a small positive rotation, showed either no optical activity or a slight levo rotation. Other investigators, notably Siebert (3) and Rost, Franz, and Weitzel (9), also failed to find definite and consistent positive rotations in urines containing benzoyl glycuronic acid. Since it seemed probable that the positive rotation recorded by Magnus-Levy, and by Brugsch and Hirsch (8), might be accounted for by the presence of free glycuronic acid which had been liberated by hydrolysis, the stability of benzoyl glycuronic acid was investigated. It was found that when a sample of urine which had a negative rotation was rendered slightly alkaline with sodium carbonate, no change took place immediately, but that in a short time it became dextrorotatory and within 3 to 5 hours had reached a maximum rotation. Roughly, the same result was obtained when the urine was rendered slightly alkaline, heated to boiling, and then quickly cooled. Under these conditions not only did the rotation become positive, but the amount of free benzoic acid greatly increased, indicating quite definitely the ease with which benzoyl glycuronic acid is hydrolyzed in an alkaline solution. An explanation for the reduction of an alkaline copper solution by benzoyl glycuronic acid is suggested, for the boiling alkaline solution must cause an immediate hydrolysis with the liberation of glycuronic acid which exerts the reducing action. It is

a question whether the specific rotation of sodium benzoyl glycuronate is correct.

In acid solution benzoyl glycuronic acid seems to be much more stable. In a sample of urine to which enough sulfuric acid had been added to make it 0.1 N, no change in rotation was observed for 24 hours. Some specimens of urine which had been kept for 2 months became dextrorotatory, although they were acid. Further study of the hydrolysis and the optical rotation of this compound is desirable.

#### SUMMARY.

A direct method for the determination of hippuric acid is outlined.

The production of hippuric acid in the organism of the dog in response to ingested benzoic acid is relatively small and comparatively fixed in amount. Exogenous glycine either free or combined causes a definite but small increase in the amount of hippuric acid excreted.

The amount of benzoyl glycuronic acid excreted is variable depending upon the dose of benzoic acid ingested. When large amounts of benzoic acid are fed the glycuronic acid conjugation becomes by far the most important mechanism.

Benzoyl glycuronic acid is very unstable in alkaline solution and it appears to be either optically inactive or slightly levorotatory.

*Addendum.*—Recently a small quantity of crystalline material which is probably benzoyl glycuronic acid has been isolated. It is slightly levorotatory in aqueous solution and dextrorotatory in ethyl acetate. Further work on this compound will be reported later.

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## THE BASAL METABOLISM OF GIRLS.

BY KATHARINE BLUNT, JENNIE TILT, LAURA McLAUGHLIN,  
AND KATHERINE B. GUNN.

(From the Department of Home Economics, University of Chicago, Chicago.)

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For several years past the authors have been making observations on the basal metabolism of children, chiefly from the University Elementary and High Schools. Data have now accumulated on 46 girls, and approximately the same number of boys. The material on girls is presented in this paper. With most of the subjects duplicate observations were made on two closely consecutive days usually on or near the birthday; with 22 girls the study has been repeated a 2nd year, and with 15, a 3rd, a total of 153 days observations. Many of the girls' results collected in 1922 and 1923 were sent to Dr. Francis G. Benedict who commented upon them in his paper before the American Philosophical Society (1).

### EXPERIMENTAL PROCEDURE.

*The Girls.*—The children range in age from 8, the youngest when the first measurements were taken, to 18 at the latest; most were from 9 to 13 years. All had been medically examined by the school physician and in general reported in good health. The height and nude weight of the girls were secured the 1st year from the medical record made on or near the child's birthday; the 2nd year, from the weight in underwear corrected by average observed weights of the underwear of 25 of the girls; and the 3rd year, from weights in ordinary clothing with merely shoes and outer garments removed corrected as recommended in the Baldwin and Wood tables (2). The differences introduced by these different procedures are very slight. Thus all weights in the tables are *nude weights*.

For comparison of the weight and height of the children with normals the Baldwin and Wood tables, published in 1923, are

used throughout, the older observations, made before these were available, being recalculated. The weight averages of these tables take into account both height and age; the height averages are for age and are divided into medium (the average of the middle 75 per cent of the 12,000 girls measured), short, and tall. In our calculations of height variation of our children we have used the figure for medium as a basis of comparison. It should be noted that height variation expressed in percentage of average height gives a much smaller figure than weight variation; 10 per cent under- or overweight is that counted as significant in this paper and 4 per cent under- or overheight.

The cooperation of the children was usually satisfactorily secured. Through the interest and support of the school officials, particularly Prof. H. C. Morrison, superintendent of the Laboratory Schools, and Principal H. O. Gillet of the Elementary School, the children and their parents were led to consider the test merely as part of the school routine.

*Management.*—The children came for the observation late in the morning, the parents having been asked to give an early breakfast with no meat, eggs, or coffee, and the children warned to eat nothing during the morning. They were always questioned on these points, and the very few times when there was reason to feel doubt about them the observation was not made or, if made, was discarded. This late hour rather than the much more difficult early morning was considered justified by the observations of Bauer and Blunt (3) that a light breakfast had no effect upon the metabolism of children 4 hours afterwards. The children were excused from gnasium work the morning of the experiment and all other active exercise discouraged.

A half hour rest period, lying quietly on the couch, always preceded the observation, and the importance of absolute quiet was impressed upon the child. The agreement commonly obtained between the first and second 10 minute observations was proof that complete rest had been attained. For the 1st years a story was read out loud during the rest and experiment, but the last year this was abandoned, with equally satisfactory results.

Except in very warm weather a light blanket was thrown over the child who wore ordinary indoor clothing, and the room was always comfortably heated.

*Apparatus.*—Some form of the Benedict portable respiration apparatus was used throughout, an early Sanborn, a Collins, and the last year a Roth (4) modification with the flutter valves in place of the blower. The last 2 years, tracings of the respirations were made on a kymograph and marked by a timer; earlier, ocular readings of the scale and two stop watches were used. Precautions were always taken to test for leaks in the apparatus, at first merely before the observations; and later when the kymograph was used, by watching for a change in the slope of the tracings when a weight was placed on top of the spirometer in the midst of an observation. Unabsorbed carbon dioxide was also tested for by passing a sample of the residual gas after an observation into barium hydroxide. This was done less frequently in the earliest experiments than later, but the almost uniformly negative results in this as in the test for leaks give reassurance as to the correctness of the earlier technique.

For a time a pneumograph was attached to the bottom of the springs of the couch and tracings from it recorded on the kymograph; these always agreed exactly with the observer's own reports as to a child's movements.

The determinations the 1st year were made chiefly by J. Tilt, the 2nd by L. McLaughlin, and the 3rd by K. B. Gunn. J. Tilt worked with the senior author in final organization of the material.

*Computations.*—In all computations the modifications suggested by Carpenter and Fox (5) were used, the earlier results obtained before the publication of Carpenter and Fox's paper being recomputed. That is, the empirical correction of 1.8 mm. per degree rise in temperature for change in volume of the air in the apparatus during the experiment was made directly on the observed volume of oxygen consumed, and, as Wilson soda lime was employed throughout, the gas was considered saturated with water vapor, or else, with the Roth apparatus, his 2 per cent correction was made. Carpenter's tables (6) were of constant usefulness.

The main possible source of error in a metabolism experiment with good technique is the child's tenseness which might raise the oxygen consumption, and a low value checked by repetition is therefore to be regarded as a truer basal than an average of all observations. The matter of deciding what figures to consider as satisfactory and what to discard as obviously inaccurate was

simplified in this series by the fact that for most children observations were taken on 2 days close together. If the two periods on these 2 days agreed within 5 per cent the average was called the basal metabolism. If the agreement was not so good, it was decided, on the advice of Dr. Benedict, to take as basal the two lowest periods of the 2 days if these checked. A few cases where the 1st day's oxygen consumption was in both periods consistently higher than the 2nd day's, are included in the table, but the calculation of calories is made only from the 2nd day's value.

#### RESULTS AND DISCUSSION.

The results of the metabolism determinations are given in Table I, and a summary of the material, giving averages for the different ages, is brought together in Table II. For the five ages from 9 to 13 there is a sufficient number of cases to justify drawing tentative conclusions from the average; at the other ages, with only two or three subjects each, an unusual case, as the two large 15 year old girls, has too great influence on the average. The noticeable features of the 9 to 13 year average figures (Table II) are the steady increase in total calories with age, from 1084 to 1437; the almost equally regular decrease of calories per kilo, from 37 to 30; and the much slighter and somewhat irregular differences in the calories per square meter and per cm. It is remarkable that for these five ages the average calories per square meter vary from an average of 42.5 by only  $\pm 4$  per cent; and that the average calories per cm. vary from an average of 8.57, by only +7 and -5 per cent. Plainly height and surface have more to do with rate of metabolism than has age or weight directly.

Our few values for 14 years and above, when height increases have begun to slow up, show a decided decrease in calories per square meter, and less regularly in calories per cm.

*Individual Variations.*—When the variation of individual children from the average of their age is considered, it is also observed that these two methods of comparison, for surface, and for height, give somewhat closer values than do total calories and calories per kilo, though the advantage is not so marked as when the group averages are compared. The individual 9 year olds, for example, vary on the average from their average calories per

TABLE I.  
*Fundamental Data.*

Subject No	Date.	Age.		Weight.	Height.	Pulse rate.	Oxygen* per min.	Calories.			
		yr.	mo.	kg.	cm.		cc.	Per 24 hrs.	Per kilo 24 hrs.	Per cm. 24 hrs.	Per sq. m. 1 hr.
1	Nov., 1922	7	10	31.9	130	80	166, 160	1133	35.7	8.71	44.5
1	May, 1923	8	4	32.1	131		166, 161	1139	35.5	8.70	44.7
2	Mar. "	8	4	28.4	131	83	168, 164	1153	40.6	8.81	47.6
Average of two† 8 year old girls.....								1144	38.1	8.76	46.1
1	Oct., 1923	8	10	35.4	136		156	1084	30.6	7.98	40.7
2	Nov., "	9	0	31.9	137		165	1146	36.0	8.38	43.1
3	" 1922	8	6	24.9	128	100	143	986	39.6	7.70	43.7
3	May, 1923	9	0	26.7	130		140, 140	973	36.4	7.47	41.3
4	Feb., 1924	9	0	29.5	131	79	152, 155	1070	36.2	8.15	42.9
5	Apr., "	9	0	32.6	131	86	183, 179	1258	38.6	9.63	48.5
6	" "	9	0	25.5	127	72	155, (167)	1077	42.3	8.50	46.7
7	Oct., 1922	9	0	26.8	128	81	143	989	36.9	7.75	42.9
8	Apr., 1923	9	0	29.2	133	75	149, 144	1015	34.8	7.55	41.1
9	Nov., "	9	1	35.8	141	69	178	1237	34.5	8.76	43.3
Average of ten 9 year old girls.....								1084	36.6	8.19	43.4
1	Jan., 1923	10	0	40.5	145		199	1383	34.2	9.54	45.0
3	Apr., 1924	10	0	28.7	135	73	150, 156	1063	38.8	7.91	42.2
7	May, 1923	9	7	28.0	130	70	161, 166	1139	40.7	8.75	47.5
10	Mar., 1924	10	0	29.6	139	70	180, 176	1237	40.8	8.90	47.7
11	Apr., "	10	0	34.3	134	70	(181), 166	1153	33.6	8.61	42.9
12	" "	10	0	32.1	142	76	(180), 168	1167	36.3	8.22	42.0
13	" "	10	0	30.0	135	70	158, 150	1070	35.6	7.96	42.1
14	Mar., 1923	10.	0	23.1	131	73	149	1035	44.8	7.90	45.9
15	Nov., "	10	0	31.0	133	90	173	1202	38.7	9.06	46.9
16	Dec., 1922	10	0	36.0	142	90	163, (176)	1133	31.4	7.98	39.3
17	Jan., 1923	10	0	35.2	136	81	179, 177	1237	35.1	9.07	45.1
Average of eleven 10 year old girls.....								1165	37.2	8.53	44.2
16	Nov., 1923	11	0	39.0	150	72	(176), 156	1084	27.7	7.25	34.7
17	Dec., "	11	0	36.3	142	84	(198), 186	1292	35.5	9.11	44.1
18	Feb., "	11	0	31.5	139	92	161, 164	1133	36.0	8.16	42.5
19	Oct., 1922	11	0	31.8	142	92	173	1202	37.8	8.48	44.7
20	Apr., 1924	11	0	28.9	134	80	187, 188	1306	45.1	9.76	52.3



TABLE I—Continued.

Subject No.	Date.	Age.		Weight. kg.	Height. cm.	Pulse rate.	Oxygen* per min.  cc.	Calories.			
		yr.	mo.					Per 24 hrs.	Per kilo 24 hrs.	Per cm. 24 hrs.	Per sq. m. 1 hr.
21	Jan., 1923	11	0	37.3	140	87	(179), 164	1139	30.5	8.13	39.6
22	" "	11	0	48.9	151	80	(208), 182	1265	25.8	8.38	36.8
23	Oct., 1922	11	0	36.2	148	90	168	1167	32.2	7.91	39.2
24	Feb., 1924	11	0	29.7	140	87	166, 165	1146	38.5	8.17	43.8
25	Apr., 1923	11	0	35.5	145	71	186, 182	1278	36.0	8.79	44.4
26	Jan., "	11	0	36.5	141	84	163, 163	1133	31.0	8.06	39.6
27	Apr., "	11	0	41.3	146	80	171, 168	1181	28.5	8.08	38.4
28	" "	11	0	33.2	141	89	161, 162	1119	33.7	7.94	40.5
Average of thirteen 11 year old girls.....								1188	33.7	8.32	41.5
15	June, 1925	11	8	37.9	141	81	(195), 185	1285	33.9	9.11	43.9
16	Nov., 1924	12	0	47.8	160	70	218, 214	1501	31.4	9.38	42.8
17	Dec., "	12	0	40.6	147	73	194, 194	1348	33.2	9.10	44.2
18	Feb., 1924	12	0	37.1	150	68	172, (188)	1195	32.2	7.99	39.5
19	Nov., 1923	12	0	34.4	147	79	(180), 156	1084	31.5	7.37	38.9
21	Jan., 1924	12	0	44.3	144	80	192, 186	1313	29.6	9.12	41.7
23	Oct., 1923	12	0	38.6	153	77	173	1202	31.1	7.86	41.7
25	Apr., 1924	12	1	40.5	155	76	214, 210	1473	36.3	9.53	46.1
26	Jan., "	12	0	39.1	148	72	188, 192	1320	33.7	8.92	43.3
27	Apr., "	12	0	48.8	158	85	218, 209	1487	30.4	9.41	43.0
28	Mar., "	12	0	33.9	145	80	171, 170	1200	35.4	8.30	42.4
29	Dec., 1922	12	0	57.1	156	87	221	1536	26.9	9.82	40.7
30	Jan., 1925	11	7	34.7	149	87	195, 193	1348	38.8	9.05	46.0
31	Feb., 1923	12	0	27.7	136	78	153, 142	987	35.6	7.28	38.4
32	Jan., 1924	12	0	52.3	159	84	200, 197	1376	26.3	8.70	37.9
33	" 1923	12	0	49.9	147	77	205, 203	1417	28.4	9.64	42.2
34	Apr., "	12	0	36.4	139	87	187, 193	1320	36.2	9.49	46.6
35	Mar., "	12	2	34.4	143	85	169, 167	1167	33.9	8.16	41.9
Average of eighteen 12 year old girls.....								1309	32.4	8.74	42.3
19	Nov., 1924	13	1	37.7	151	98	191, 197	1348	35.7	8.91	43.9
23	" "	13	0	40.8	160	92	188, 188	1306	32.0	8.16	39.7
25	Apr., 1925	13	0	46.7	163	69	(228), 207	1438	30.7	8.80	40.4
26	Jan., "	13	0	47.6	154	85	226, 227	1570	32.9	10.2	46.0
27	Apr., "	13	0	51.9	159	77	195	1355	26.1	8.52	37.4
28	Mar., "	13	0	37.2	149	78	169, 166	1160	31.2	7.78	38.6
29	Dec., 1923	13	0	65.0	161	78	231, 231	1605	24.6	9.96	39.8

TABLE I—*Concluded.*

Subject No.	Date.	Age.		Weight.	Height.	Pulse rate.	Oxygen* per min.	Calories.			
								Per 24 hrs.	Per kilo 24 hrs.	Per cm. 24 hrs.	Per sq. m. 1 hr.
		yr.	mo.	kg.	cm.		cc.				
31	Feb., 1924	13	0	33.3	142	83	163, (181)	1133	33.7	7.98	40.6
32	Jan., "	13	0	56.0	165	84	230, 236	1619	28.9	9.81	42.1
33	" "	13	0	57.7	157	73	240, 240	1681	29.1	10.7	44.6
36	Feb., 1923	12	6	36.3	151	85	(170), 150	1070	29.4	7.08	35.4
37	Mar., 1924	13	0	53.2	159	78	260, 261	1813	35.9	11.4	49.0
38	Dec., "	13	0	60.0	159	74	269, 258	1834	30.6	11.4	48.1
39	Nov., 1922	13	1	36.8	152	78	151	1049	28.5	6.90	34.4
40	Apr., 1923	13	5	67.1	159	80	228, 225	1570	23.4	9.86	38.0
Average of fifteen 13 year old girls .....								1437	30.2	9.16	41.0
31	Feb., 1925	14	0	39.3	147	90	190, 188	1313	33.4	8.93	43.0
32	Jan., "	14	0	53.9	169	90	220, 221	1529	28.3	9.00	39.6
39	Apr., 1923	13	6	39.0	156	71	179	1244	31.9	7.33	39.2
39	Oct., "	14	0	43.4	161	60	195	1355	31.2	8.41	40.0
Average of three† 14 year old girls.....								1380	31.1	8.60	40.7
29	May, 1925	14	6	71.6	164	70	209, 206	1447	20.2	8.82	33.8
41	" 1923	15	5	66.4	168	72	223, 224	1549	23.3	9.20	36.7
Average of two 15 year old girls.....								1498	21.8	9.01	35.3
41	May, 1924	16	4	62.0	171	70	(228), 215	1494	24.1	8.74	35.4
42	" 1923	16	5	43.6	160	90	164, 165	1139	26.1	7.12	33.6
Average of two 16 year old girls.....								1317	25.1	7.93	34.5
42	May, 1924	17	5	43.4	162	69	(190), 167	1160	26.7	7.20	34.3
43	" 1923	16	11	49.2	161	61	188, 187	1299	26.4	8.06	36.1
44	" "	16	11	53.2	164	74	185, 182	2171	23.9	7.77	33.9
Average of three 17 year old girls.....								1543	25.7	7.68	34.8
42	Dec., 1924	17	11	43.0	163	93	196, 195	1355	31.5	8.34	39.8
45	May, 1923	17	6	57.0	164		210, 211	1459	25.6	8.93	37.5
46	" "	18	1	70.3	169	64	203, 200	1403	20.0	8.30	32.9
Average of three 18 year old girls.....								1406	25.7	8.51	36.7

\* Where two values for oxygen are given they were obtained on different days usually within a week of each other. Except for the figures enclosed in parentheses, the average of the oxygen figures is used in computing the calories.

†Subjects 1 and 39 are each counted as one child only because the two observations were just under 6 months apart. Other similar cases with two observations each are counted as two children because the observations were 6 months or more apart.

square meter by  $\pm 4.0$  per cent, and from their average total calories by  $\pm 7.2$  (see Table III).

This variation of our individual children from the average for their age is rather slight at the younger ages, but increases up to the 13 year group, when there is just about twice as much variation as at 9 years. The increase at 13 years is probably due to the fact

TABLE II.  
*Average Results for Each Age Group.*

Age.	No. of subjects.	Calories per 24 hrs.	Calories per kilo per 24 hrs.	Calories per sq. m. per hr.	Calories per cm. per 24 hrs.
8	2	1144	38.1	46.1	8.76
9	10	1084	36.6	43.4	8.19
10	11	1165	37.2	44.2	8.53
11	13	1188	33.7	41.5	8.32
12	18	1309	32.4	42.3	8.74
13	15	1437	30.2	41.0	9.16
14	3	1380	31.1	40.7	8.60
15	2	1498	21.8	35.3	9.01
16	2	1317	25.1	34.5	7.93
17	3	1543	25.7	34.8	7.68
18	3	1406	25.7	36.7	8.51

TABLE III.  
*Average Variations of the Individual Values from the Averages for Each Age and for Each Method of Comparison.*

Age.	No. of subjects.	Average percentage deviation $\pm$ .				Sum of the four averages.
		Total calories.	Calories per kilo.	Calories per cm.	Calories per sq. m.	
9	10	$\pm 7.2$	$\pm 6.0$	$\pm 5.8$	$\pm 4.0$	23.0
10	11	$\pm 6.3$	$\pm 8.4$	$\pm 5.7$	$\pm 5.3$	25.7
11	13	$\pm 5.2$	$\pm 12.1$	$\pm 5.3$	$\pm 8.1$	30.7
12	18	$\pm 8.8$	$\pm 8.5$	$\pm 7.4$	$\pm 5.1$	29.8
13	15	$\pm 14.5$	$\pm 9.5$	$\pm 13.2$	$\pm 8.5$	45.7

that this group is much less homogeneous than the younger ones, less nearly normal in weight and height; it contains eight girls who were more than 10 per cent over or under the Baldwin and Wood standard of weight, and nine who were more than 4 per cent over or under the average height, most of these being on the tall and heavy side of the standards. Of the 9 year old group only

two were unusual in weight (overweight) and one in height. This difference is probably chiefly merely the result of accident in selecting the children but partly of real physiological significance, for the "range of variation within what may be considered normal limits [of weight and height] is greater beyond the ages of twelve and thirteen years than at earlier ages" (Baldwin and Wood).

*Under- and Overweight Girls.*—Of all of our 9 to 13 year old girls, eleven (ten individuals, one of whom was observed a 2nd year) were more than 10 per cent underweight for their height and age. Every one of these showed some sort of decided variation from the average for her age. Six had markedly high calories per kilo, eight had markedly low total calories, and three had both of these irregularities at once. On the average (Table IV) these eleven girls varied from the averages for their age by  $-11.4$  per cent for

TABLE IV.  
*Variations of Underweight and Overweight Girls from the Average for Their Ages.*

	No. of girls.	Average percentage variation in:				
		Weight.	Calories per 24 hrs.	Calories per kilo.	Calories per cm.	Calories per sq. m.
Underweights.....	11	-13	-11.4	+6.0	-10.9	-3.9
Overweights.....	13	+18	+10.3	-11.3	+9.0	0.0
1921 underweights.....	9	-19	-3.2	+19.9	-1.4	+6.6

the total calories, and  $+6.0$  per cent for calories per kilo; *i.e.*, they were low if the total calories are considered and slightly high for the calories per kilo. On the other hand our thirteen overweights (counting three twice on their 2 years) tended to have high total calories (averaging  $+10.3$  per cent deviation), low calories per kilo ( $-11.3$  per cent), and high calories per cm. ( $+9.0$  per cent). Comparison on the basis of calories per square meter brings both groups close to their averages.

Thus the girl of average build can be compared with our average for her age fairly satisfactorily by any one of our four methods of comparison, with only a slight advantage for surface or height, but the over- or underweight girl *only on the basis of calories per square meter*.

In 1921, the senior author of this paper, with Nelson and Oleson,

(7) reported figures showing the high basal metabolism of two groups of underweight children. Nine of these were girls from 9 to 13 years of age. The results have been recomputed taking the closely agreeing minima as recommended by Benedict, rather than averages of all observations, and compared with the average observations for similar ages in this paper. The figures are included in Table IV. It is noteworthy that the earlier children, considerably more underweight than the present ones, are very high in calories per kilo, much higher than the underweights of this paper, and also higher in the three other methods of comparison, though for these three methods fairly close to the average for the whole present group. It looks as if the underweight girl

TABLE V.  
*Average Percentage Variations from Benedict's Standards.*

Total calories compared with height.			Calories per kilo compared with age.		
Age.	No. of girls.	Variation from Benedict's standard.	Age.	No. of girls.	Variation from Benedict's standard.
8	2	+17.7	12	18	+4.4
9*	10	+9.9	13	15	+7.2
10*	11	+13.2	14	3	+17.7
11†	13	+9.1	15	2	-8.1
			16	2	+15.1
			17	3	+16.2
			18	3	+17.9

\* Standards taken partly from curve, extrapolation of table.

† All but three standards taken from curve.

were metabolizing at not far from the same rate as a child of the same height and age and *normal* weight, as if the fat she should have were on her body, or as if she had her normal amount of active protoplasmic tissue. Whether or not she is considered to have a high metabolism depends upon the standard of comparison used. If calories per kilo, she tends to be high; if total calories or calories per cm., she tends to be normal or low.

*Comparison with Benedict's Standards.*—Benedict (1) has suggested two standards for girls, one on the basis of height up to 138 cm., and the other on the basis of calories per kilo, from 12 to 18 years (8, 9). All but one of our 11 year girls and several of our 10 year olds were taller than 138 cm., so that no standard was

available for them, and to get a standard for comparison we have read from the curve which Benedict has drawn continuing the slope past 138 cm. At all ages, with one exception not worthy of much consideration, and for the great majority of our individuals we run higher than Benedict (Table V), as he has already pointed out (1). The average excesses for our five ages with most of our cases vary from 4.4 per cent to 13.2 per cent.

Benedict and Talbot's (10) earlier standard for girls under 12 which was based on a weight rather than a height comparison, fitted underweights much less satisfactorily than the present one. The nine underweights of Blunt, Nelson, and Oleson's paper average 16 per cent above the old Benedict and Talbot standard and only 8 per cent above the present. On the other hand, fat children, or those unusually tall for their age, agree less

TABLE VI.  
*Comparison with MacLeod.*

Age.	No. of subjects.		Calories per 24 hrs.		Calories per kilo per 24 hrs.		Calories per sq. m. per hr.		Calories per cm. per 24 hrs.	
	Mac-Leod.	This paper.	Mac-Leod.	This paper.	Mac-Leod.	This paper.	Mac-Leod.	This paper.	Mac-Leod.	This paper.
11	4	13	1191	1188	29.2	33.7	37.5	41.4	7.90	8.32
12	18	18	1295	1309	32.3	32.4	41.3	42.4	8.61	8.74
13	22	15	1365	1437	31.1	30.2	41.0	41.0	8.87	9.17
14	13	3	1308	1380	26.7	31.1	36.6	40.8	8.23	8.69

well with the new standard, both of these groups, as might be expected, tending to run high.

Our figures also show a number of variations from Benedict's standard, chiefly on the high side, and from our own averages, which cannot be explained on the basis of abnormal build. The basal metabolism of children is not so simply prophesied.

*Comparison with MacLeod.*—Miss Grace MacLeod of Teachers College, Columbia University, has published an extensive series of observations on girls 11 to 14 years old (11). Dr. Benedict had access to her figures before publication as well as to ours, and spoke in his Philosophical Society address (1) of their being in close agreement with ours and higher than his.

We have brought together in Table VI MacLeod's figures and ours for her four ages, including total calories, and calories per

kilo, square meter, and cm. We both have a fairly large number of 12 and 13 year old children, and for these ages our figures are remarkably close by all four methods of comparison. Of 11 year subjects she has only four, and of 14 year olds we only 3; so that conclusions have to be drawn only very tentatively. It is just at these ages that we agree least well. She has higher calories per kilo, square meter, and cm. at 12 and 13 years than before or after. We do not show any such break in our curve; our calories per kilo and per square meter decrease fairly regularly with age. Our curve of calories per cm. is nearer hers.

Nine of our girls are known to have commenced to menstruate at some time during one of the years when their metabolism was observed. These are Nos. 18, 19, 27, 29, and 32 at 12 years old, and Nos. 33, 37, 38 at 13 years, and No. 39 at 14 years. No consistent change can be noted in their year-to-year variation in metabolism. The year for starting was higher for some girls than the year preceding and lower for others. Thus neither our average figures for our different ages, nor the variation in our few individual girls, show any effect of puberty.

Bedale (12) in England has also observed the basal metabolism of young girls varying in age from 9 to 17. Her figures for her youngest and her oldest children agree fairly well with ours, but for her 11 and 12 year groups, they are much higher.

#### SUMMARY.

1. Figures are presented for the basal metabolism of 46 girls varying in age from 8 to 18. On twenty-two of the girls the determination was repeated a 2nd year and on fifteen a 3rd. From ten to eighteen girls were observed at each of the ages from 9 to 13, justifying the taking of averages and the setting up of tentative standards for these ages.

2. From 9 to 13 years the total calories per 24 hours increase steadily from 1084 to 1437, the calories per kilo decrease almost equally regularly from 36.6 to 30.2, and the calories per square meter per hour from 43.4 to 41.0. The calories per cm. fluctuate around 8.1 to 9.2. By the two latter methods of comparison based on surface area and height, the averages for these five ages are remarkably close to each other.

3. Girls of build different from the average tend to have a basal metabolism different from the average.

4. Underweight girls tend to show a high basal metabolism if computed in terms of calories per kilo, a low or normal metabolism in terms of total calories or calories per square meter or per cm. For overweight girls the figures are reversed: the calories per kilo are low and the total calories and the calories per cm. high.

5. Comparisons made on the basis of calories per square meter seem to show the least variation from the average for different individuals, whether of *normal* or *unusual build*.

6. Our results are almost uniformly higher than the standards for girls proposed by Benedict—a fact already pointed out by him from consideration of our figures before publication.

7. Most of our results are in close agreement with MacLeod's, especially on the total calorie basis. At 12 and 13 years we agree with her almost exactly on all four of our methods of comparison, but at 11, and with very limited data at 14, our calories per kilo and per square meter and per cm. are higher than hers. We thus agree with her that the previously proposed standards for girls of 12 and 13 are too low, but do not agree that those for 11 and possibly 14 are too high.

The authors wish to express to Dr. Francis G. Benedict their appreciation of his helpful suggestions in reading and criticizing this paper.

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# THE EFFECT OF SLEEP ON URINARY CHLORIDES AND pH.

By GEORGE ERIC SIMPSON.

*(From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia, and the Department of Biochemistry, McGill University, Montreal, Canada.)*

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## INTRODUCTORY.

During sleep the volume of urine is low, in the case of normal individuals. The chemical composition of the urine shows certain changes. It is desirable to know whether these two variations bear any relation to each other. It will be shown that it is possible to prevent the change in volume, and that characteristic chemical changes (low pH and low chloride excretion) occur, nevertheless.

The data to be presented may also be regarded from a somewhat different point of view. There is evidence for an interrelationship between urinary pH and the rate of urine excretion in the case of 24 hour samples. Sleep has been used, in this work, as a means of obtaining urines of low pH values. It is shown that no marked change in volume necessarily accompanies the variation in pH which occurs in 2 hour samples under these conditions.

It has been shown that, even though the amount of water ingested per hour is constant during the 24 hours, the volume of urine excreted per hour during the night is low, nevertheless (Simpson, 1924).

Watson (1923-24, 1925), and Bazett and coworkers (1924) reported an increase in urinary pH following waking from sleep in the morning. Hubbard, Munford, and Allen (1924) did not usually find this increase in pH on waking from sleep, nor did Campbell (1920) usually find an alkaline tide in the morning unless a meal was ingested. In experiments in which urine samples were collected hourly for 24 hours by the writer, low values for urinary pH were found during sleep at night.

Experiments which indicate that chloride excretion is low during sleep at night have been reported by Chaussin (1912), Campbell and Webster (1921, 1922), Kleitman (1923), and the writer. It seemed probable, upon studying these results, that the low nocturnal excretion of chloride might be secondary to the smaller volume of urine usually found during sleep.<sup>1</sup> Bazett and coworkers found that both the concentration of urine and the rate of chloride excretion were increased after waking from sleep in the morning in many of their experiments. Marked fluctuations in urine volumes are to be seen in the experiments reported by them.

In a comparison of 24 hour samples of urine, pH and volume showed a definite relationship (Henderson and Palmer, 1914). In statistical studies of short interval urines, Hubbard and Munford (1922) found slight evidence of any correlation between pH and volume; later, Hubbard (1925) found no correlation whatever.

#### EXPERIMENTAL.

In order to suit the purpose for which these experiments were originally planned, it was considered desirable to promote diuresis on the day preceding the experiment. Accordingly, the subjects (male students) were asked to drink large amounts of water and an extra cup of coffee for breakfast and lunch on this day. Supper was omitted; an enema was taken at about 5 p.m. Thereafter the subjects drank 200 cc. of water each hour until the collection period started when they retired about 10 p.m. Throughout the experiments the subjects remained in bed, and the ingestion of 200 cc. of water at 37–40° each hour was continued. Urine was collected at regular intervals of 1 or 2 hours, as indicated in the tables.

Chlorides were determined by the Volhard procedure without separation of the silver chloride precipitate except in the first experiments. Phosphates were determined by Briggs' (1922) modification of the Bell-Doisy procedure. Recovery of a known amount of phosphate, added to an appropriate quantity of urine, with an error usually less than 1 per cent was the criterion of correct technique. pH was determined colorimetrically, following Marshall's (1922) suggestions as to collection of samples.

<sup>1</sup> This does not apply to Kleitman's experiments on himself (Kleitman, 1923). Kleitman behaved differently from most normal individuals in that he excreted larger volumes of urine during sleep than when he was awake; moreover, he was shown to be "slightly nephropathic" in his response to the phthalein test.

## DISCUSSION.

Six experiments in which the subjects slept for varying lengths of time during the night are reported in Table I. These show that the changes in chloride excretion associated with waking from sleep in the morning are of considerable magnitude. In all experiments the rate of chloride excretion increased at least threefold after waking.

Control experiments were carried out in which the subjects stayed awake at night and slept in the morning, at the time the above mentioned increases would otherwise have been expected. These are reported in Table II. Chloride excretion did not show any marked change in these experiments. It was slightly depressed during the morning sleep in two of the five experiments; in the other three the rate was either practically unchanged or it increased slightly.

The men selected for the experiments claimed to be good sleepers.

With reference to the experiments reported in Table II, it cannot be said that the subjects were wide awake during the entire night (although their eyes were never closed) nor can it be said that all the subjects were always sound asleep from 7 to 11 a.m. The difficulty in determining the condition of the subjects will be appreciated. In two experiments in which urinary pH and chlorides increased in the morning, the subjects surely slept well: vigorous shaking was necessary to waken MA at the hourly intervals; RAB waved his arms to protect his eyes from the light and muttered incoherently when the sleeping room was entered for the purpose of carrying out the usual hourly routine. After the experiment he did not recollect that he had been awakened between 7 and 11. Although JFL seemed to sleep as well as either MA or RAB, no clear-cut decrease in chloride excretion was seen during the morning sleep in this experiment. That HUH and MAD were disturbed by the entrance of the caretaker at the hourly intervals was indicated by their moving about in bed.

If it had been possible for all five subjects to be wide awake until 7 a.m., and sound asleep from that time on, a decrease in chloride excretion during the sleep would probably have been obtained in every case.

The opposite effect from that shown on waking from sleep in the morning in Table I—that is, a decrease in chloride excretion on falling asleep at night—has been found in many experiments.

TABLE I.

*Effect of Waking from Sleep on Urinary pH and Chloride Excretion.\**

Time.	Subject JFL-1.					Subject LOH.				
	Volume.	pH	Cl	Phosphate.	Remarks.	Volume.	pH	Cl	Phosphate.	Remarks.
a.m.										
1						432	6.1	4.28	1.25	Awake.
2										Sleep.
3	125	6.3	0.62	0.91	Sound sleep.	250	6.2	4.35	0.92	"
4					" "					"
5	280	6.4	1.08	1.89	" "	228	6.1	4.21	1.06	"
6					" "					"
7	200	6.3	1.02	1.09	" "	250	6.5	8.34	1.02	Awake during
8	206	6.4	1.59	0.93	" "	295	7.8	15.84	1.19	rest of ex-
9	215	6.5	3.71	1.08	Awake.	465	7.0	16.98	0.88	periment.
10	215	7.1	4.86	0.97	"	220	7.3	19.21	1.04	
11	410	6.9	7.25	0.94	"	295	7.0	13.64	1.10	Headache.
12	268	7.0	4.92	1.09	"	270	6.9	15.84	1.31	Slight head-
p.m.										ache.
1	236	6.9	6.14	1.44	"	225	6.7	13.90	1.05	Headache.
2	237	6.8	5.00	1.38	"	330	6.7	15.20	1.41	No headache.
3	250	6.5	3.24	1.26	Sleep $\frac{1}{2}$ hr.	200	6.3	10.38	1.24	" "
4	185	6.4		1.37	Awake.	200		8.38	1.23	" "
5	270	6.5		1.40	"	100	5.7	9.66	0.66	" "
6						96	5.4	5.98	0.61	" "
Time.	Subject VM-1.					Subject RAG.				
	Volume.	pH	Cl	Phosphate.	Remarks.	Volume.	pH	Cl	Phosphate.	Remarks.
a.m.										
1	415	6.0	4.63	0.88	Awake.					
2					Sleep.					
3	237	6.0	4.31	0.67	"	215	5.9	5.3		Sleep.
4					"					"
5	167	6.5		0.64	"	220	6.0	6.8		"
6					Awake.					"
7	375	6.8	7.74	0.53	"	225	6.2	9.5		"
8	305	7.9	19.75	0.55	"	235	6.6	13.6		Awake.
9	405	7.2	15.46		"	257	6.8	14.4		"
10	185	7.0	13.32	0.60	"	207	6.8	11.7		"
11	150	6.9	10.97	0.78	Headache.	280	6.4	11.7		"
12						150	5.7	6.9		"
p.m.										
1						187	5.7	7.5		"
2						130	5.4	3.6		"
3						240	5.6	4.9		"
4						442	5.9	4.3		Sleep.
5						245	5.7	5.5		" $\frac{1}{2}$ hr.
6						120	5.6	6.4		Awake.

TABLE I—*Concluded.*

Time.	Subject TB.				Subject UL.			
	Volume.	pH	Cl	Remarks.	Volume.	pH	Cl	Remarks.
a. m.								
1								
2								
3	170	5.7	2.1	Sleep	337	6.7	4.55	Sleep.
4				Wakeful.				"
5	232	6.1	2.8	Sleep.	327	6.7	7.24	Wakeful.
6				"				"
7	215	6.4	4.1	Awake.	325	7.0	12.52	Awake.
8	375	6.8	9.1	"	310	8.0	20.10	"
9	380	6.8	12.7	"	307	7.3	15.26	"
10	185	6.9	12.8	"	255	7.1	12.26	"
11	245	6.2	12.1	"	120	7.1	11.57	Headache.
12	165	5.6	9.0	"	80	6.5	7.79	No headache.
p. m.								
1	112	5.7	6.9	"	165	5.7	7.59	Sleep.
2	135	5.7	4.3	"	125	5.4	5.66	"
3	202	5.7	4.1	"	100	5.4	4.39	"
4	200	5.7	2.8	"	117	5.6	3.69	"
5	135	5.5	2.6	Sleep.	142	5.5	3.20	"
6	20	5.4	0.7	"	90	5.4	2.32	"

\* Chlorides and phosphates in millimols. These, and volume (in cc.), all as units excreted per hour for the 1 or 2 hour periods.

One of these was shown in Chart 2 of the previous paper. Marked diminution in the amount of urine excreted per hour usually accompanied these decreases. From many experiments a few may now be selected in which the volume changes are either lacking entirely or are very slight. In these experiments, a marked diminution in chloride excretion occurs at the onset of sleep (Table III).

Phosphate excretion is apparently not markedly affected by sleep. The hourly excretion is higher in the afternoon or late morning than in the early morning. The results are similar to those reported by Fiske (1921).<sup>2</sup>

<sup>2</sup> The recently published results of Kleitman (1925) who collected urine in 2 periods during the 24 hours—a sleep period and a "wakefulness" period—are interpreted by Kleitman as disagreeing with this conclusion.

The low pH during sleep is in consonance with the results reported by Leathes (1919), which may be interpreted as showing an increase in the alkalinity of the urine after waking from sleep

#### Effect of a Morning Sleep on Urinary pH and Chloride Excretion.\*

[illegible]

TABLE II—*Concluded.*

Time.	Subject UH.				
	Volume.	pH	Cl	Phosphate.	Remarks.
<i>p. m.</i>					
11					
12					Awake.
<i>a. m.</i>					
1	300	6.9	2.36	0.63	"
2					"
3	205	6.9	3.45	0.72	"
4					"
5	350	7.1	4.28	0.68	"
6					"
7	390	7.2	4.06	0.59	"
8	360	7.0	4.44	0.69	Sleep.
9	155	7.1	4.76	0.76	"
10	280	6.9	5.22	1.06	"
11	145	7.0		1.03	"
12					

\* Chlorides and phosphates in millimols. These, and volume (in cc.), all as units excreted per hour for the 1 or 2 hour periods.

in the morning. It is also in consonance with the high level of ammonia and titratable acid excretion during sleep which has been reported by many workers (Osterberg and Wolf, 1907; Campbell and Webster, 1921, 1922; Fontés and Yovanovitch, 1923; the writer; Bazett *et al.*, 1924; and others). The urine thus approaches in its composition that which is characteristic of uncompensated CO<sub>2</sub> excess in the sense of Van Slyke (1921). The composition of the blood in sleep is also of this type. H<sub>2</sub>CO<sub>3</sub> concentration is high (Straub, 1915; Leathes, 1919; Bass and Herr, 1922), bicarbonate concentration is practically unchanged (Collip, 1920), and the pH is low (Endres, 1923). A decreased excretion of chlorides during sleep might therefore be expected on the basis of a shift of chloride from serum to corpuscles. Gollwitzer-Meier and Kroetz (1924), however, have reported an increase, rather than a decrease, in the concentration of chloride in the serum during sleep. If these findings are confirmed, the low level of chloride excretion must be ascribed to changes in renal permeability or renal activity during sleep.



TABLE III.  
*Effect of Falling Asleep at Night on Chloride Excretion. Selected Experiments.\**

Subject MeI.				Subject D. Raf.†				Subject Stat.‡			
Time.	Volume.	Cl	Remarks.	Time.	Volume.	Cl	Remarks.	Time.	Volume.	Cl	Remarks.
<i>p.m.</i>				<i>p.m.</i>				<i>p.m.</i>			
4-6	192	4.6	Awake.	-6	155	14.3	Awake.	5-7	125	0.98	Awake.
-8	247	5.0	"	-8	260	22.7	"	-8	90	0.88	"
-10	162	4.9	"	-10	170	17.6	"	-9	80	0.70	"
-12	150	2.8	Sleep.	-12	147	12.4	Sleep.	-10	150	0.45	Light sleep.
<i>a.m.</i>				<i>a.m.</i>							
-2	185	1.7	"	-2	158	10.9	"	-11	175	0.24	Sleep.
-4	147	1.7	"	-4	117	11.0	"	-12	75	0.14	"
								<i>a.m.</i>			
-6	172	1.9	Awake ½ hr.	-6	162	10.0	"	-1	95	0.15	"
-8	135	2.2	"	-8	137	14.8	Awake.	-2	100	0.16	"
-10	235	2.8	"					6-7	165	0.30	Awake.
Subject GCP-2.§				Subject NBH-2.§				Subject W. W.			
<i>p.m.</i>				<i>p.m.</i>				<i>p.m.</i>			
2-4	182	6.2	Awake.	2-4	300	5.2	Awake.	3-4	178	11.5	Awake.
-6	250	6.1	"	-6	370	6.2	Dozed ½ hr.	-5	183	9.2	"
-8	154	5.9	"	-8	266	7.4	Awake.	-6	174	"	"
-10	161	4.0	"	-10	200	9.3	"	-7	328	13.3	"
-12	163	1.9	Sleep.	-12	145	5.9	Sleep.	-9	155	8.0	"
<i>a.m.</i>				<i>a.m.</i>							
-2	179	1.7	"	-2	171	"	"	-10	220	9.8	"



*Effect of Sleep during the Day on Urine Volume.*

It is well known that urinary volume is low during sleep at night. It is, nevertheless, extremely difficult to demonstrate any effect of natural sleep during the day time on urinary volume.

To date, thirty-five experiments of 24 hours duration have been carried out by the writer in which the activities of the subjects were regulated as described above and in which the experimental period proper began after a night's sleep in the laboratory. The subjects of these experiments slept during the day when they desired. Periods of sleep occurred during the day in seventeen of the thirty-five experiments, but a change in urine volume which was a result of the sleep is clearly evident in only one instance (Table IV). This single experiment showing a clear-cut positive result is reported for two reasons. In the first place, experiments in which prolonged natural sleep occurs during the day time with the subject under rigid experimental

TABLE IV.

*Effect of Sleep during the Day on Urinary Volume.\**

Time ....	-9 a.m.	-10 a.m.	-11 a.m.	-12 m.	-1 p.m.	-2 p.m.	-3 p.m.	-4 p.m.	-5 p.m.	-6 p.m.	-7 p.m.	-8 p.m.	-9 p.m.	-11 p.m.	-1 a.m.	-3 a.m.	-5 a.m.	-7 a.m.
Volume .	200	400	305	155	<b>260†</b>	<b>25</b>	39	222	236	131	269	142	<b>73</b>	<b>112</b>	215	<b>260†</b>	<b>135</b>	<b>180</b>

\* Figures in bold type indicate periods of sleep.

† Asleep 20 minutes.

‡ Asleep 1st hour, awake 2nd hour.

control will probably continue to be rare. In the second place, the reasons for the lack of positive results in the other experiments are evident. The naps were usually of short duration (in many instances less than 1 hour,—the collection period of urine). Furthermore, the naps occurred chiefly in the afternoon. But it is practically impossible to ascribe to sleep any decrease in volume which may occur at this time. The reason for this is that experiments in which there are no day time naps always show a marked decrease in volume sometime in the afternoon—as early as 2 and as late as 6 p.m. (Simpson, 1924)<sup>3</sup>.

<sup>3</sup> It is the intention of the writer to report further from results which are still accumulating, on the extent of the variations to be found in short interval urine volumes when the experimental men are subjected to the limited physical activity of such experiments as these.

In a group of nine of the sixteen experiments, it was quite clear that sleep had no effect on the urine volume curve; one subject slept 1.5 hours, another 3 hours, a third 0.9 hours, the remaining six slept 0.5 hours or less. In a second group of three experiments, there was a possible decrease in volume as a result of sleep: one subject slept 2 hours; the other two subjects, each  $\frac{1}{2}$  hour or less. In two other experiments, urine volumes were somewhat large during the sleep periods of 0.5 and 1.0 hours respectively. The urine volume curves in the remaining two of the sixteen experiments exhibited extreme irregularity, but it was not possible to say of any variation that it was an effect of sleep. In one of these, the subject slept three times, for 0.25, 0.5, and 0.75 hours at a time; in the other the subject dozed 2 hours and slept 3 hours between 1 and 6 p.m.

The writer gratefully acknowledges his indebtedness to the medical students at McGill University and the University of Pennsylvania who served as the subjects of these experiments. The data of Tables I and II were obtained in Philadelphia; of Tables III and IV, in Montreal. This work was aided by a grant from the Cooper Memorial Fund of McGill University.

#### SUMMARY.

1. On waking from sleep in the morning, urinary pH and the amount of chloride excreted per hour showed marked increases. These were not the result of changes in urine volume.

2. When the subjects stayed awake until 7 a.m. and slept from 7 to 11, the matutinal increases were lacking.

3. In certain experiments, selected because of the absence of significant changes in volume, it was possible to demonstrate a decrease in chlorides and pH on falling asleep at night.

4. Among seventeen experiments in which natural sleep occurred during the day time, one showed an unmistakable effect of sleep on urine volume. In this, a marked decrease in volume occurred as a result of the sleep. The reasons for the lack of positive results in the other experiments are given in the text.

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## THE IRON CONTENT OF MEATS.\*

By E. B. FORBES AND RAYMOND W. SWIFT.

*(From the Institute of Animal Nutrition of the Pennsylvania State College,  
State College.)*

(Received for publication, December 26, 1925.)

Reawakened interest in the iron content of meat products has followed the very productive series of researches by Whipple, Hooper, and Robscheit-Robbins which have demonstrated, with the dog as a subject, among other points, "that the curve of hemoglobin regeneration can be influenced at will by various diet factors" (1); that liver, heart, and skeletal muscle "were most potent in effecting a rapid production of hemoglobin and red cells" (2); that "beef liver feeding in severe anemia is associated with a maximal regeneration of hemoglobin and red cells" (3); and that this is by no means simply an effect of the iron of the food though "long continued severe anemia due to hemorrhage may be associated with iron depletion and these experiments in dogs show a favorable reaction to iron treatment . . . ." (4).

A late paper by Hart and associates (5) has also contributed to our interest in the iron content of meats by materially extending our understanding of the method of participation of the iron of foods in the prevention and cure of anemia.

While it is well known that meats constitute an important source of iron in the diet, so little reliable information on the iron content of meats is on record that Sherman, in his text-book, *Chemistry of Food and Nutrition* (6), submits no detailed data, as with vegetable foods, but proposes the use of an average ash analysis for "meat," and another for "fish," as applying to 100 gm. of protein, in all cases.

With the thought of learning how nearly iron comes to varying in meats directly as the protein, as thus indicated, and therefore

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how great the need for individual iron determinations on meats, this study was undertaken.

The explanation of the remarkable paucity of information on this subject doubtless lies in the unusual difficulty involved in the preparation of samples of meat free from iron contamination.

In the solution of this problem we enjoyed the kindly cooperation of the Enterprise Manufacturing Co. of Philadelphia who assisted us in finding an unusually hard phosphor-bronze, and in casting a large knife, and making a power-driven meat chopper, entirely from this virtually iron-free alloy. Through the use of these mechanical facilities no unusual difficulties were experienced in the preparation of thoroughly good samples, the validity of which was attested by the entirely satisfactory agreement of triplicates, which could hardly have been contaminated, from the mechanical equipment, in a perfectly uniform manner.

The meats were all purchased in the local market, and were trimmed in a manner intended to approximate the condition in which they are ordinarily eaten. All portions which could have come in contact with a steel knife, or other sources of contamination, were cut away, with the bronze knife, and the entire portion, usually of several pounds, was then ground through the bronze chopper into a porcelain dish.

A glance at the column of ether extract figures (Table I) shows that these were unusually lean meats; and a comparison of these data with the analyses of the same kinds and cuts of meat as reported in Office of Experiment Stations Bulletin 28, shows that, in general, our samples were of the fatness there designated "very lean."

Portions of about 50 gm. each, for iron estimation, were weighed out into porcelain dishes at the time of grinding, and were covered with watch-glasses and put into a cold storage room, where they remained, in a frozen condition, until the estimations could be made. Samples for ether extract, moisture, and nitrogen determination were also taken at the time of preparation. All estimations represent at least three agreeing determinations.

The samples for iron estimation were ignited to a white ash, and dissolved in hydrochloric acid, the iron being determined by titration with  $\text{KMnO}_4$  solution which was about 0.005 normal. The triplicate iron estimations all agreed to 0.0001 per cent.

In the ashing of meats trouble often results from spattering,

especially if the samples contain a high percentage of fat. While most of our samples were low in fat, the bacon contained 64.10 per cent, and all were ashed without any spattering, by first drying the samples in an electric oven, and then ashing in an electric muffle furnace, with careful control of heat with the aid of a thermoelectric pyrometer.

At the beginning of the study the ashing was performed in porcelain dishes, but these were abandoned in favor of silica

TABLE I.  
*Composition of Meat Samples.*

Sam- ple No.	Kind of meat.	Iron.	Nitro- gen.	Protein (N $\times$ 6.25).	Ether extract.	Moisture.	Mg. iron per 100 gm. of protein.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
105	Bacon cured.	0.0013	1.67	10.44	64.10	22.31	12.5
107	Ham "	0.0014	3.37	21.06	13.60	64.61	6.6
114	Pork shoulder.	0.0015	2.93	18.31	14.41	67.14	8.2
108	" hind quarter.	0.0015	3.14	19.63	10.54	69.07	7.6
112	Lamb shoulder.	0.0016	3.01	18.81	6.94	72.95	8.5
113	" hind quarter.	0.0016	3.25	20.31	5.27	75.59	7.9
102	Beef rib.	0.0024	3.24	20.25	6.05	72.70	11.9
100	" round.	0.0025	3.55	22.19	3.48	74.65	11.3
101	" loin.	0.0025	3.29	20.56	6.39	72.02	12.2
103	" chuck.	0.0025	3.17	19.81	7.13	72.84	12.6
109	Veal fore quarter.	0.0023	3.47	21.69	1.70	76.08	10.6
110	" hind "	0.0027	3.54	22.13	1.54	76.68	12.2
116	" kidney.	0.0040	2.61	16.31	4.17	77.82	24.5
111	Beef heart.	0.0044	2.59	16.19	4.94	78.91	27.2
117	" brain.	0.0053	1.68	10.50	7.89	79.15	50.5
104	" liver.	0.0082	3.30	20.63	4.66	68.33	39.7
115	" spleen.	0.0138	3.03	18.94	1.90	77.50	72.9
106	" kidney.	0.0188	2.58	16.13	1.85	78.67	116.6
118	" blood.	0.0444	2.87	17.94		80.99	247.5

dishes, when it was observed that those samples of meat ash which fused removed the glaze from the dishes. This porcelain glaze, being transferred with the sample into the beaker in which the titration was made, gave a milky appearance to the solution, and rendered the end-point indistinct and difficult to catch. Platinum was also found to be quite impracticable.

Consequently, all determinations which had been made in porcelain dishes were repeated in dishes of silica. The end-points



where silica dishes were used were definite and distinct. The agreement with the estimations made in porcelain, however, was better than anticipated. Slightly higher results, about 0.0002 per cent, were obtained in nearly every determination ashed in a silica dish. This is probably due to an involuntary tendency to anticipate the end-point in solutions where it is not well defined. The end-points with samples ashed in silica dishes were as sharp as in distilled water.

The different kinds of meat differed greatly in their tendency to attack the dish during ashing; and this applies not only to porcelain but also to silica, which was sometimes etched. Those meats exhibiting the greatest tendency to etch the dishes were spleen, ham, and liver, while beef heart, beef loin, and lamb were entirely without effect to roughen the glaze of the dish.

When the samples of beef blood were ignited in silica dishes there remained in the dish, after boiling with concentrated HCl, a reddish-brown insoluble residue which could not be dissolved or scoured off. This was found to be iron. Fresh samples were ignited below redness, and extracted with HCl. The residue was then fully ignited, and added to the HCl extract which had been evaporated to dryness and also ignited, to eliminate the possibility of organic matter being present. By this procedure we avoided the insoluble combination of iron and silica dish. In general, an extraction which removes the readily soluble salts from the partially ashed meat prevents the dish from being attacked by the remaining constituents of the ash.

The potassium permanganate solution used was equivalent to approximately 0.275 mg. of Fe per cc., and was standardized, from time to time, with sodium oxalate. The  $\text{KMnO}_4$  was carefully prepared, and the apparatus used was so arranged that the solution was never exposed to circulating air, or to dust. The first standardization, on June 3, showed a strength of 0.3007 mg. of Fe per cc. The last standardization, on October 20, showed a strength of 0.2940 mg. of Fe per cc. A half dozen standardizations, performed at more or less regular intervals between these dates, show that the slight loss in strength was a gradual one. In computing the iron in the meat samples the strength of  $\text{KMnO}_4$  used was that obtained in the most recent standardization. In the case of a sample containing about 0.0025 per cent of iron it

would make a difference of only about 0.00006 per cent whether the first or last standardization was used.

An examination of the figures for iron shows a marked similarity of the iron content of muscle meat from different parts of the carcass.

Sherman's factor of 15 mg. of iron per 100 gm. of protein seems to be a little high for beef and veal, and much too high for lamb and pork, while it does not apply at all closely in relation to heart, brain, liver, spleen, kidney, and blood.

The organ meats, or "extra carcass parts," are all much richer in iron than carcass meat; indeed their richness in this important nutrient suggests that further attention should be given the utilization of these parts as human food.

Basing our comparisons with foods other than meats on the analyses compiled by Sherman—beef spleen, liver, kidney, and blood contain more iron than do any foods of vegetable origin.

Beef and veal contain two-thirds more iron than do pork and lamb, and ten times as much iron as does milk.

Beef heart and brain contain about twice as much iron as do beef and veal.

Beef liver contains twice as much iron as does beef heart.

Beef spleen contains half as much again of iron as does beef liver.

Beef contains twice as much iron as do potatoes; two and a half times as much as white flour, and corn-meal; and eight times as much iron as do apples.

Vegetable foods which contain more iron than does beef are peas, beans, lentils, graham flour, oatmeal, shredded wheat, and spinach.

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# STUDIES ON THE SYNTHESIS AND ELIMINATION OF CERTAIN BILE COMPONENTS IN OBSTRUCTIVE JAUNDICE.\*

By J. L. BRAKEFIELD AND CARL L. A. SCHMIDT.

*(From the Division of Biochemistry and Pharmacology, University of California Medical School, Berkeley.)*

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Jaundice is a condition which is usually associated with certain diseases of the liver and gall-bladder. It is characterized by the appearance of bile in the blood and urine and discoloration of the skin and sclera. The common forms of jaundice are, as regards their final etiology, brought about by stagnation of bile. The disturbance in the outflow of bile leads to its absorption within the liver and the passage of the biliary constituents into the blood and tissues induces the condition of jaundice. For purposes of experimentation jaundice may be induced in animals by ligation of the common bile duct.

The fate of the biliary constituents after passage into the blood has been the subject of a great deal of work. The literature (1) is full of contradictory statements relative to the question as to whether in icterus bile acids occur in the urine. Since most of the work was carried out on human cases in whom neither the degree of obstruction of the flow of bile nor the period of the onset of jaundice was accurately known little reliance can from a quantitative standpoint be placed in the results. Such animal experiments as were carried out were usually of short duration and at best the results are merely qualitative. There appears, however, to be sufficient evidence to warrant the statement that bile acids are present in icteric urine, at least in the early stages of the disease. The amounts, however, appear to be relatively small

\* Abstract of a thesis submitted to the Graduate Division of the University of California by James Luther Brakefield in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

and extremely variable. Certain workers have attempted to account for the paucity of bile acids in icteric urine by assuming that these substances are broken down in the blood stream and tissues but the data on the ultimate fate of the fragments are lacking. One of the inherent difficulties in attempting to account quantitatively for the fate of bile acids in jaundice is due to our lack of knowledge as regards the amount of bile acids which are daily synthesized. The data from bile fistula animals do not answer this question; they tell us how much the animal *can* synthesize under these conditions but not how much *is* synthesized by the normal animal. The bile fistula experiments of Smyth and Whipple (2) indicate quite conclusively that liver injury brought about by chloroform intoxication and certain other agencies markedly decreases the production of bile acids by fistula dogs. It is conceivable that liver injury may be brought about by biliary stasis and this may lead to a decreased production of bile acids. This aspect will be considered later.

There appears to be no good evidence contrary to the idea that synthesis of bile acids takes place wholly in the liver. On the other hand it has been quite conclusively demonstrated that bile pigments are in part formed outside of the liver (3). It is therefore not surprising to note that investigators have found bile pigments eliminated daily in the urine of old standing cases of biliary obstruction. If liver injury follows as a result of biliary stasis the amount of pigments which are found in the liver may be expected to decrease. The extrahepatic factor, however, may still contribute its quota of pigment to be excreted in the urine. It has been experimentally shown in many cases of poisoning which lead to jaundice that the production of bile acids is diminished while the formation of bile pigments may even be above normal (4). These findings may possibly also be due in part to the greater ease in the identification of the pigments when present in urine.

In considering the subject of bile acids in jaundice a number of possibilities both with respect to production and excretion suggest themselves. These are briefly enumerated: (1) They are formed in amounts equal to that of the normal animal, (2) there is a decreased synthesis, (3) synthesis does not take place. If synthesis does take place four possibilities as to the fate of

the bile acids suggest themselves: (a) They are absorbed into the blood stream and excreted quantitatively in the urine, (b) they are absorbed into the blood stream and are in part broken down and in part excreted through the kidney, (c) they are absorbed into the blood stream and are broken down completely there or in the tissues, (d) they are wholly or in part stored in the tissues.

Since no method is available for the estimation of the amount of bile acids which the normal dog synthesizes the above questions can be answered only in part. Experiments on the dog possess certain inherent advantages. This animal synthesizes only taurocholic acid. If this substance is excreted in any quantity in the urine a direct estimation is possible. If taurocholic acid is wholly or in part broken down by hydrolytic cleavage taurin should result, and judging from experiments (5) which have been carried out on the normal animal this substance is not broken down in the body but appears unchanged in the urine. Direct estimation of this substance is at the present time not possible; estimation of the neutral sulfur content of urine, however, furnishes an excellent index as to whether this substance is eliminated in any quantity.

It was the purpose of the experiments herein reported to study quantitatively for considerable periods of time the fate of the bile pigments and the bile salt in dogs in which jaundice had been produced mechanically by ligature and subsequent resection of the common bile duct. All operations were carried out under morphine-ether anesthesia on healthy dogs weighing about 15 kilos. An incision was made in the midline. The common bile duct was freed, doubly ligated and  $\frac{1}{2}$  to 1 cm. was resected. The median line was closed in the usual manner. The wounds healed promptly and within 48 hours the animals had recovered sufficiently from the effects of the operation so that urine collections were resumed. A regular routine was followed. The animals were kept in large metabolism cages in a warm room and they were fed at a definite time each morning. Water was supplied at all times. Following the collection of the 24 hour urine specimens the animals were allowed to exercise for a period of about half an hour.

The total nitrogen was estimated in the usual manner. The

total urinary sulfur was determined by the method described by Denis (6) and the total sulfate sulfur according to the Folin method (7). The neutral sulfur was calculated from the difference between the total sulfur and the sulfate sulfur. Bile acids were estimated according to the method of Schmidt and Merrill (8) and for the estimation of bile pigments the method of Hooper and Whipple (9) was employed. The estimation of the biliary constituents is no doubt less accurate than that of most quantitative procedures; for comparative purposes these methods are nevertheless useful.

In the experiments three diets were employed: (1) A low protein diet, (2) a diet identical with the above except that a certain quantity of cooked beef liver was added, (3) a diet rich in protein. The diet for a particular animal was kept as nearly constant as possible. Prior to the period of experimentation the animals were brought to nitrogenous equilibrium. Urine collections were made during a normal period and again following the operative procedure. The results are graphically given in Charts I to X.

Charts I, II, III, and IV will be discussed as a unit since they show the results of the experiments which were carried out on four dogs maintained, except for such quantitative variations as were found necessary for the needs of the individual animal, on the same diet.<sup>1</sup> It is noted that after the onset of jaundice the output of urinary sulfur increased slightly and this increase is almost wholly confined to the neutral sulfur fraction. The total and the neutral sulfur output reaches its maximum several days after the onset of jaundice and then falls more or less gradually until it reaches a value which is about the same as during the normal period. The injection of bile acids subsequent to the onset of jaundice is apparently followed by a slight rise in the sulfur output. The output of urinary nitrogen is greater after the onset of jaundice than during the normal period. Like the sulfur curve the elimination of nitrogen gradually declines and tends to approach the value for the normal. Towards the end of the experiment a slight increase in the nitrogen elimination appears to take place. Except when given in large doses the

<sup>1</sup> The diet consisted of cracker meal, Crisco, cane sugar, and condensed milk.

injection of taurocholic acid does not materially influence the excretion of nitrogen. In a general way the curves showing the elimination of bile acids are similar to the curves already discussed. The output begins to decline about a week after the

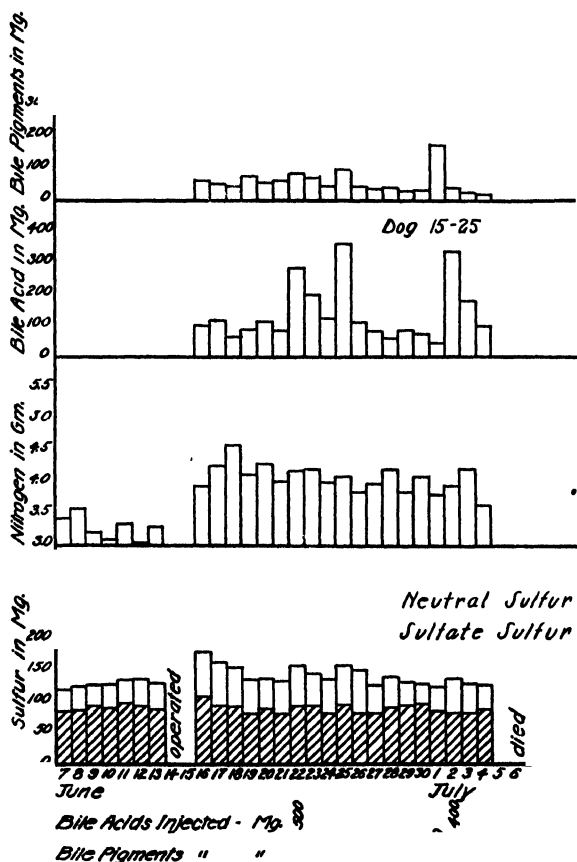


CHART I.

onset of biliary stasis and finally reaches a value which in some instances is probably within the limits of the accuracy of the method. Injection of bile acid is followed by an increase in the urinary output of this substance but the amount is not quantitative. In no instance was bile acid detected in the urine of a



normal animal after the injection of sodium taurocholate. The urinary output of taurocholic acid does not account for the whole of the neutral sulfur or nitrogen increase following biliary stasis. It is very probable that as a result of toxemia following the onset

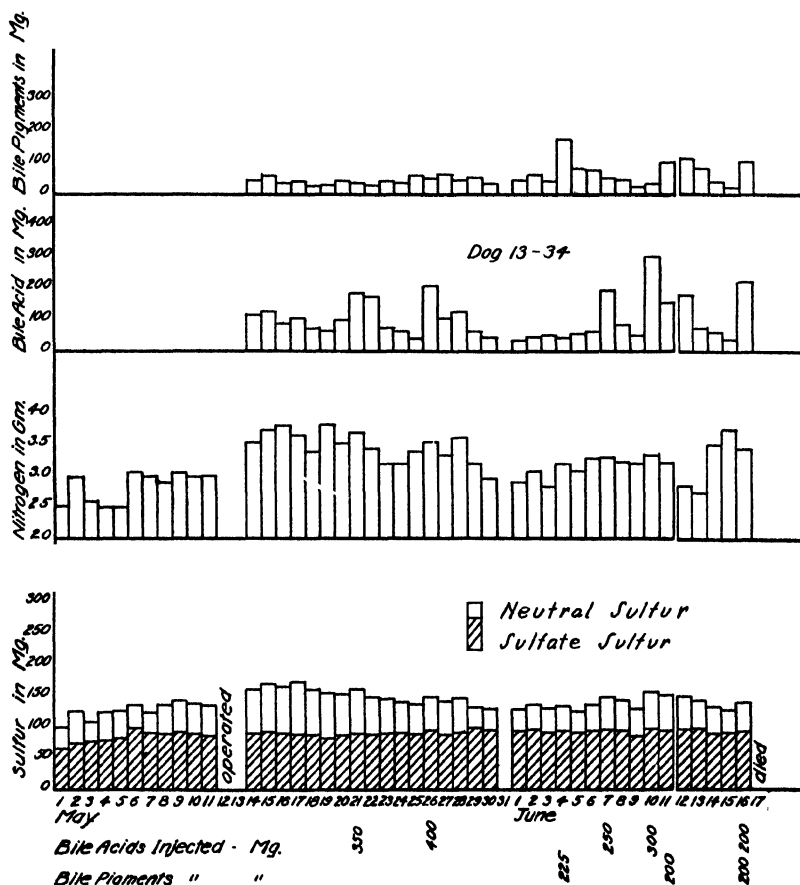


CHART II.

of jaundice a breakdown of tissue protein takes place which is expressed by a simultaneous increase of both urinary nitrogen and neutral sulfur (10). The maximum elimination of bile pigments takes place shortly after the onset of jaundice. The out-

put declines during the course of the experiment and reaches a low but somewhat constant value. Injection of bile pigments leads to a slight increase in the urinary output which continues for several days probably as the result of slow absorption.

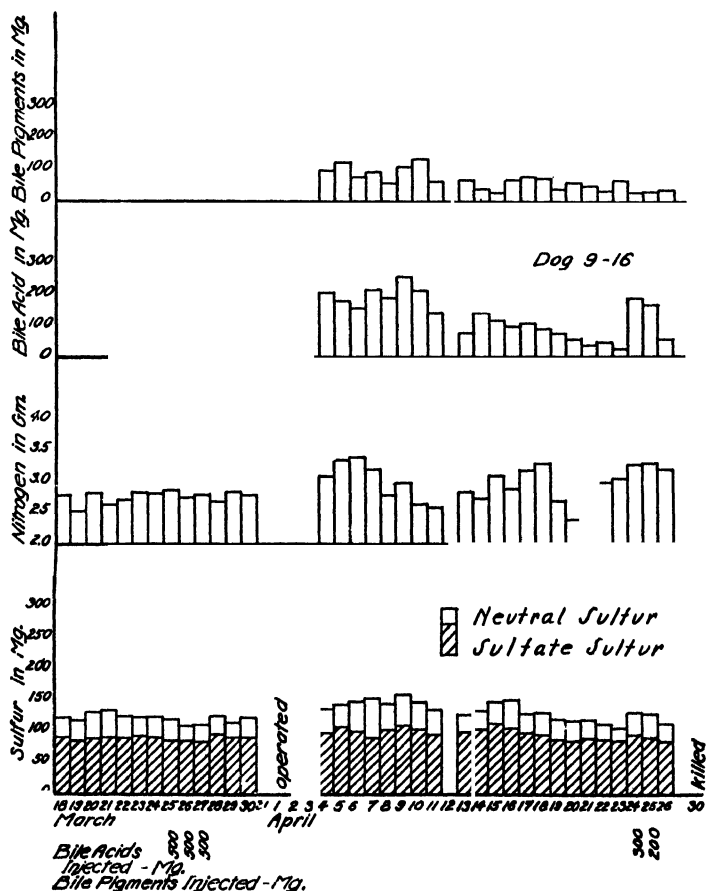


CHART III.

The diet which was used in the above experiments was low in nitrogen. Whipple and his coworkers (11) have shown that the excretion of bile acids as determined in the bile fistula dog is less on a diet which is poor in nitrogen than when the diet is

rich in nitrogen. The second series of experiments was carried out on animals whose diet differed from that of the first series by the addition of cooked liver. The results are graphically shown in Charts V, VI, VII, VIII. The results are not unlike

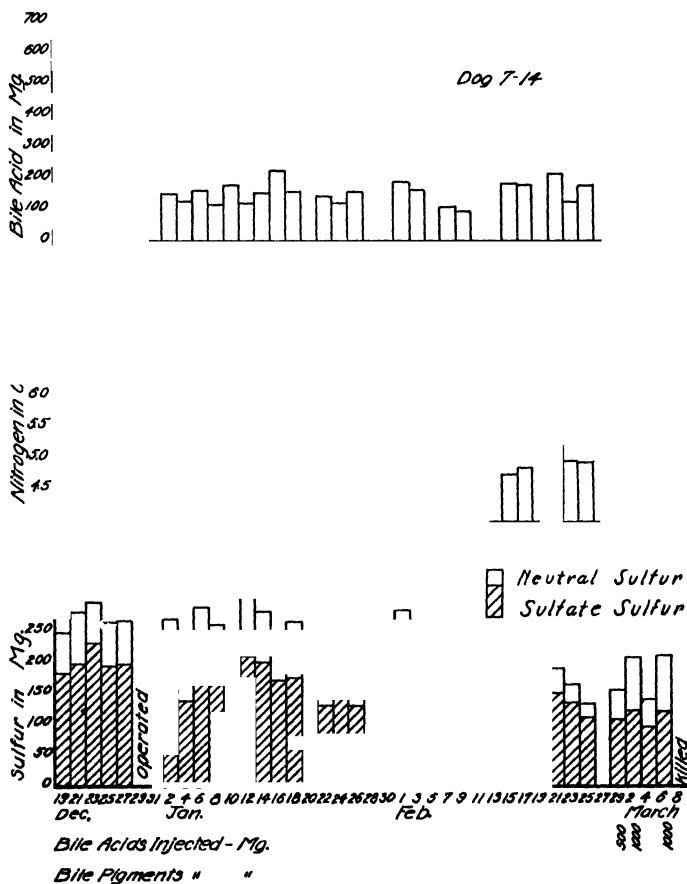


CHART IV.

those of the first series. The nitrogen output rises considerably after the onset of jaundice and after an interval of a week or more gradually declines and tends towards the normal level. Towards the end of the experiment the nitrogen output again tends to

increase. The sulfur curves simulate in a general way the curves for the output of nitrogen. The increase in sulfur output is almost entirely confined to the neutral sulfur fraction. Injection of taurocholic acid leads to a slight increase in the output of

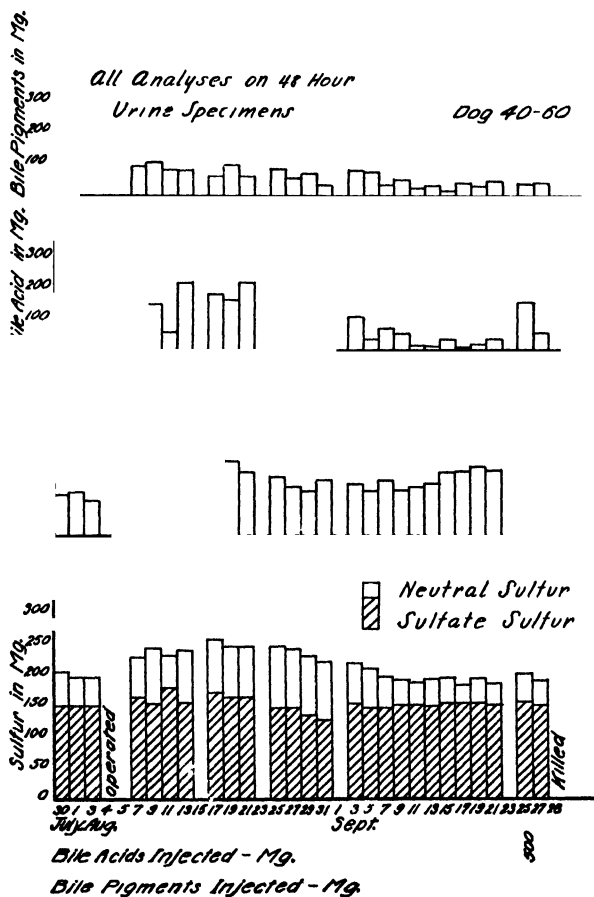


CHART V.

neutral sulfur. The output of bile acid following the onset of jaundice appears to be slightly greater in the animals of this series than in the previous experiments. The output declines after a time and reaches a very low level. Injection of tauro-

cholic acid at this stage leads to an increase in the output of bile acid. The bile pigment curves do not materially differ from those of the first series. Towards the end of the experiment a low but constant output level is reached which may be slightly

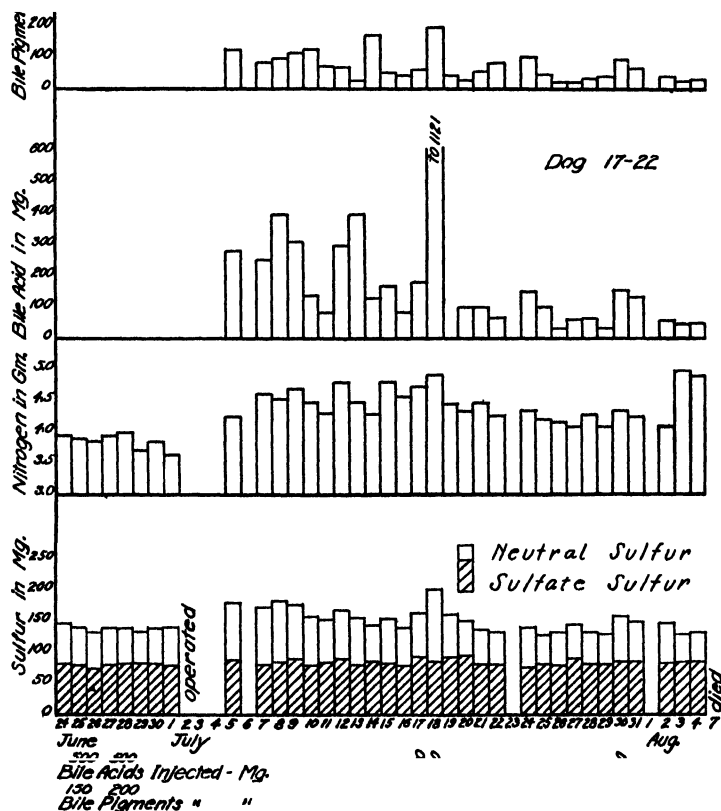


CHART VI.

increased, usually over a period of several days, on injection of bile pigments.

Two animals (Charts IX and X) were used for the experiments on a high protein diet. Dog 26-35 was fed powdered meat scraps and the other (No. 29-37) was maintained on a diet consisting

of cracker meal and powdered meat scraps to which were added 100 cc. of fresh ox bile. The difficulty due to the refusal of the animal to eat the diet was overcome by the addition of the ox bile. While the output of sulfur and nitrogen is higher in

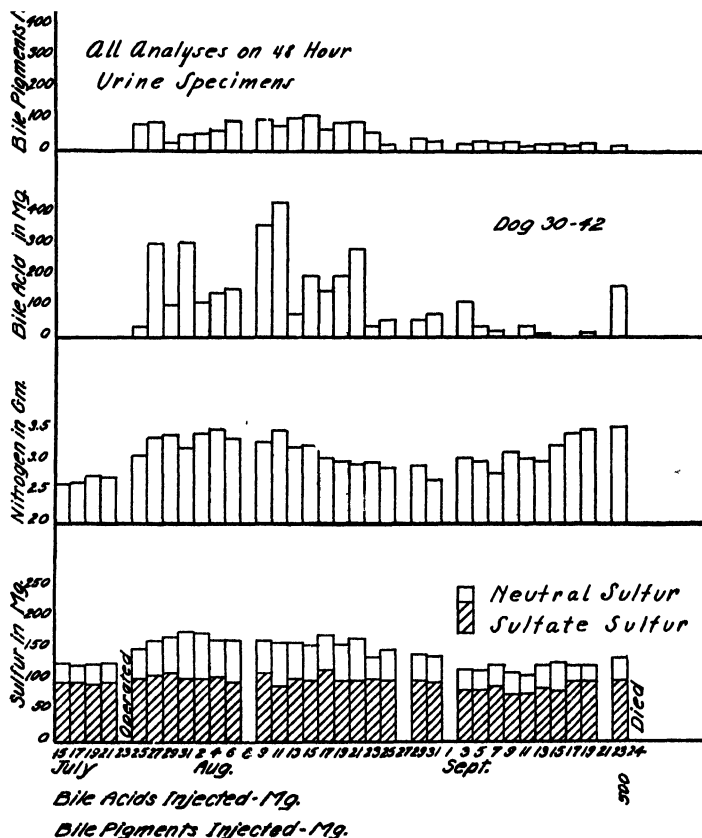


CHART VII.

these experiments than in the experiments of the other series the curves are not materially different. The increased nitrogen elimination towards the end is well illustrated in these experiments. This is probably due to tissue destruction resulting from toxemia. The sulfur curves are less marked. The magni-

tude of the bile acid output is somewhat greater in the experiments of the last two series indicating that the output is slightly influenced by the diet. There is, however, no quantitative relationship between the elimination of nitrogen, sulfur, and bile acid.

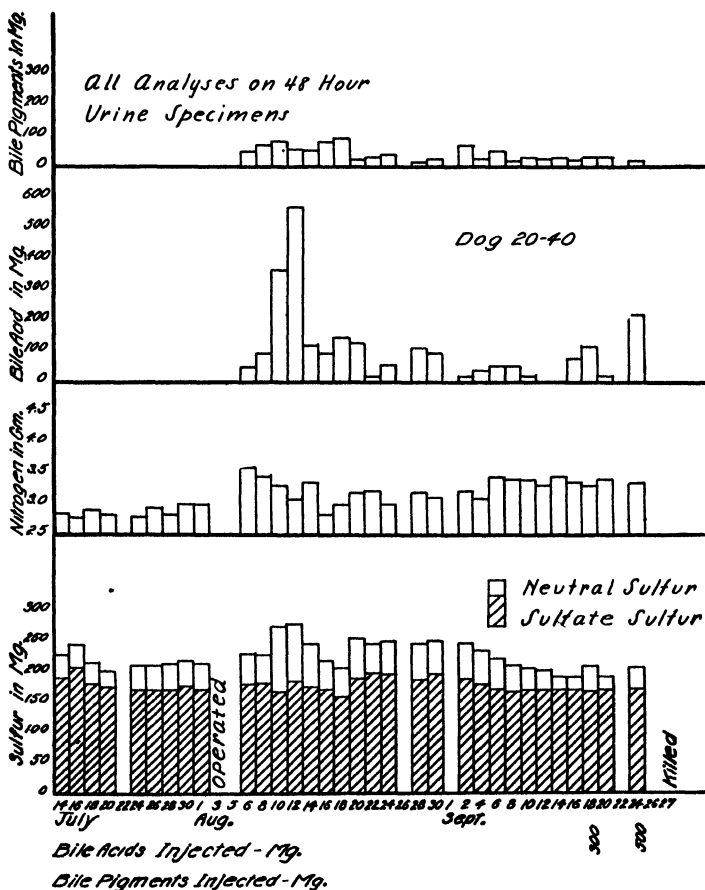


CHART VIII.

The bile pigment curves do not differ materially from those of the other series. A low and fairly constant excretory level is reached and maintained. While our experiments furnish no data, it is, however, possible that this low excretory level of bile pigment is largely of extrahepatic origin.

These experiments do not permit a balance to be made between output of bile pigments and bile acid and that which the animal

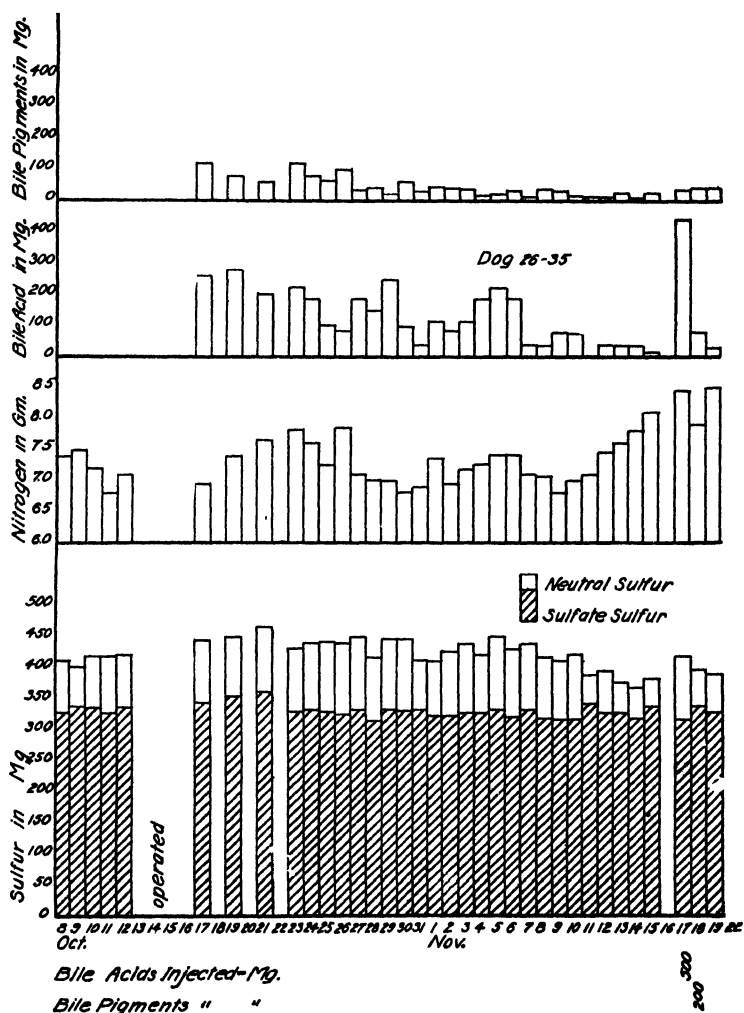


CHART IX.

synthesizes. The urinary output of bile acid represents only a small fraction of that which the bile fistula dog can synthesize.



The marked decrease in the output of bile acid some time after the onset of jaundice seemingly indicates that as a result of biliary stasis the mechanism for the synthesis of bile acids has been

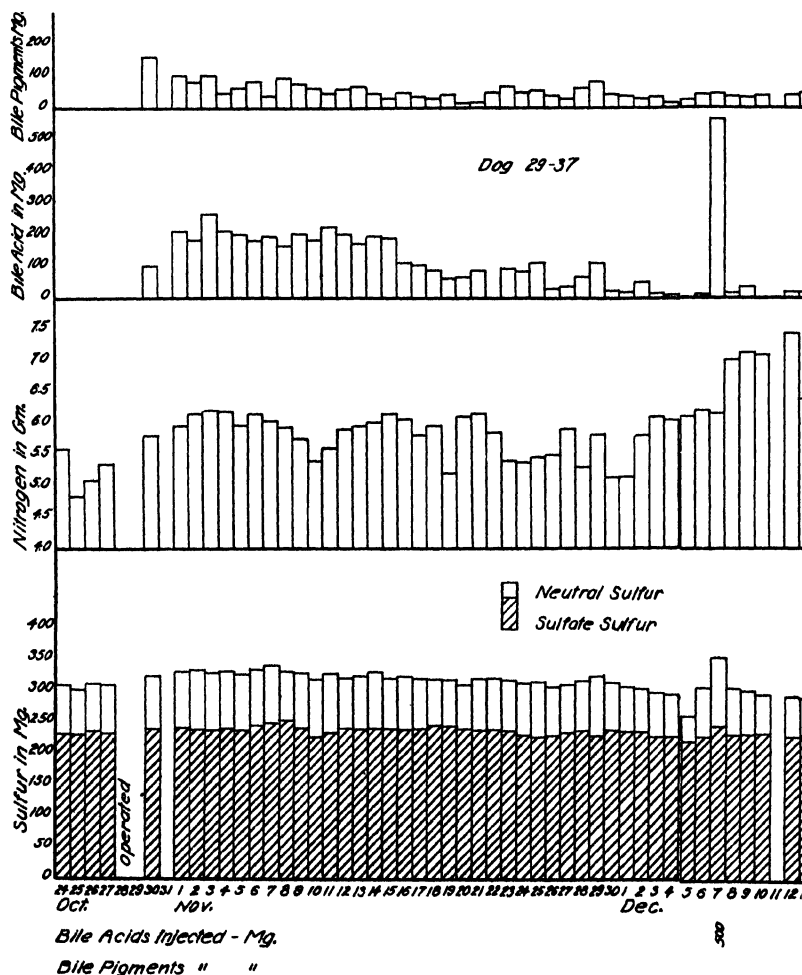


CHART X.

injured. Since it appears very probable that taurocholic acid is synthesized in the liver of the dog experiments were next carried out to determine if possible whether any impairment of

the function of the liver occurs as the result of jaundice. Mention has already been made of the experiments of Whipple and his coworkers showing that in bile fistula dogs the output of bile acid is markedly decreased by chloroform intoxication. The detoxication of benzoic acid by conjugation with glycocholl is a reaction in some ways analogous to the synthesis of taurocholic acid. A good deal of evidence has been accumulated which indicates that the liver is concerned in the detoxication of benzoic acid.

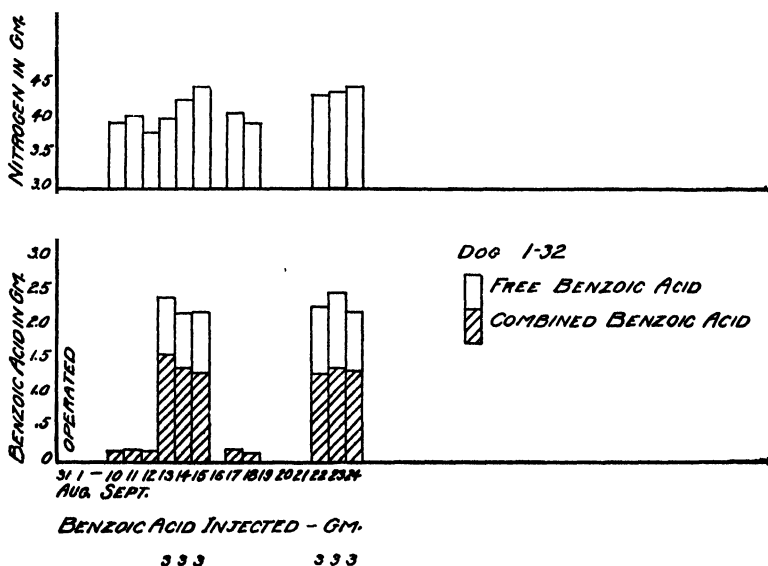


CHART XI.

Lackner, Levinson, and Morse (12) have shown that hippuric acid synthesis is decreased in animals in which liver necrosis had been produced by hydrazine sulfate. Delprat and Whipple (13) showed that synthesis of hippuric acid is not prevented by extensive chloroform necrosis but the severe injury to the liver resulted in a delayed excretion of hippuric acid. A few experiments on the synthesis of hippuric acid in jaundiced animals have been carried out but the results are not wholly uniform. Kühne (14) was unable to detect the presence of hippuric acid

in the urine of a jaundiced dog when benzoic acid was injected intravenously or given by mouth. Folwaczny (15) and others who studied human cases report that hippuric acid is synthesized by jaundiced individuals. In an experiment of short duration Lewis (16) found that a rabbit with a bile fistula was able to conjugate much less benzoic acid than normal.

Our experiments were carried out on both dogs and rabbits and the treatment of these animals with respect to operation, care,

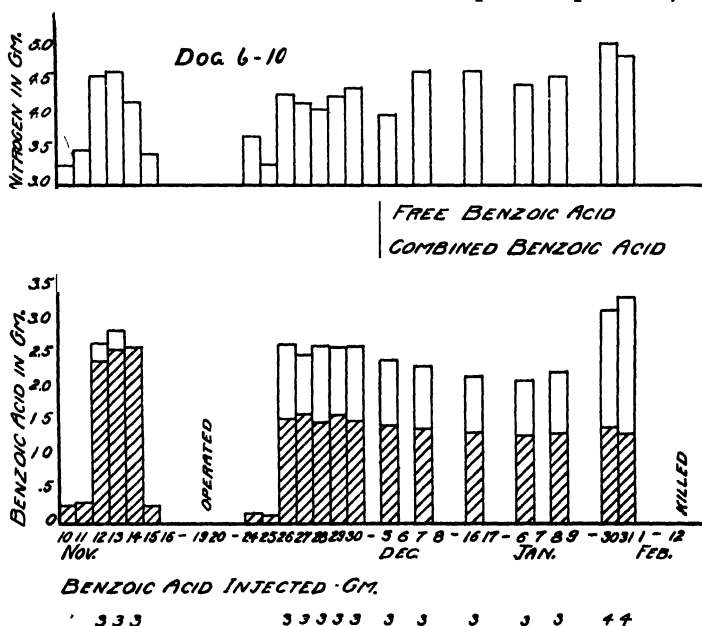


CHART XII.

and feeding was similar to that used in the previous experiments. The method of Delprat and Whipple (13) was employed for the estimation of free benzoic acid and the total benzoic acid was determined by the method of Kingsbury and Swanson (17). The difference between the values found by the two methods gives the value for the conjugated benzoic acid. In certain of the experiments a partition of the conjugated benzoic acid was further made by estimating the amount of benzoic acid which was combined with glycuronic acid. For this purpose the

method suggested by Csonka (18) was employed except that the Shaffer-Hartmann (19) instead of the Benedict reducing reagent was employed. The maximum amount of benzoic acid which the normal animal can conjugate was determined for purposes of control. The maximum dose was injected into the animal after biliary stasis had been produced and the partition of benzoic acid in the urine was again estimated.

The results of the first series of experiments which were carried out on dogs are graphically shown in Charts XI, XII, and XIII.

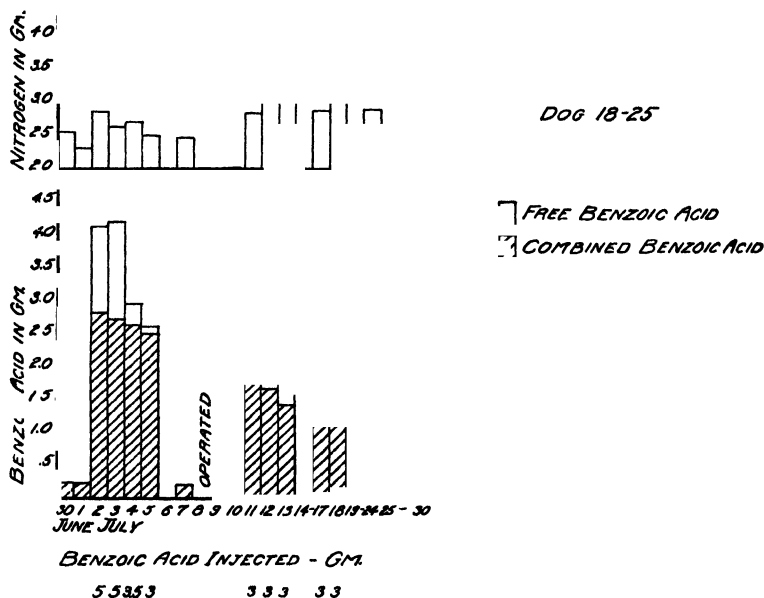


CHART XIII.

No attempt was made to determine the mode of benzoic acid conjugation. The results indicate a considerable diminution of conjugated and a corresponding increase in the excretion of free benzoic acid after the onset of jaundice. The results of the next series of experiments, Charts XIV, XV, XVI, are essentially in accord with those of the previous series. They show, moreover, that the decrease in conjugated benzoic acid can be in large part attributed to the fraction which in the normal animal is com-

bined with glycuronic acid. In this connection it is of interest to note that following ligation of the common bile duct the liver ceases to store glycogen (20). The third series of experiments (Charts XVII, XVIII, XIX) was carried out on rabbits. No attempt was made to ascertain the mode of benzoic acid conjugation. The results indicate a considerable decrease in the amount of conjugated benzoic acid after ligation of the common duct.

If it is assumed that the decrease in the output of conjugated benzoic acid is due to impairment of liver function then it must

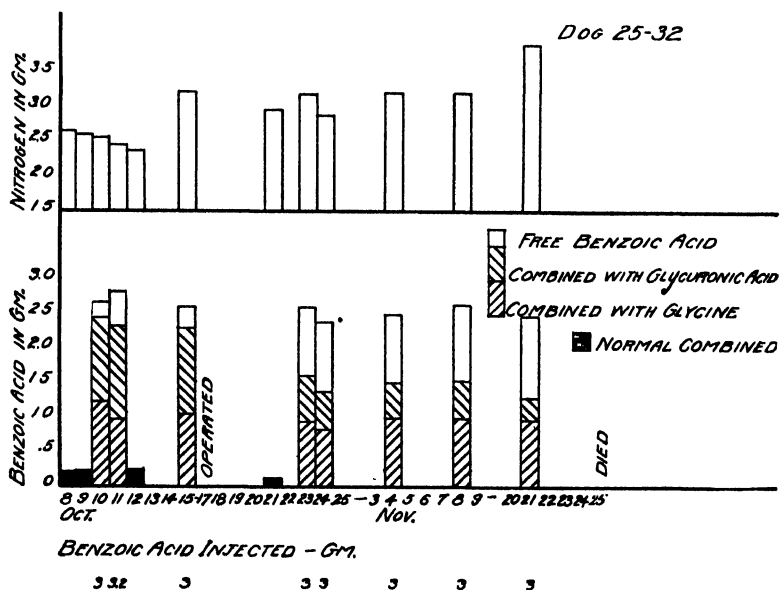


CHART XIV.

also be assumed that the liver is the chief seat for the synthesis of benzoylglycuronic acid since it is this substance whose synthesis appears to be most markedly affected. On the other hand the synthesis and excretion of hippuric acid is less affected. Whether this is indicative of the fact that this substance is synthesized chiefly elsewhere than in the liver is still a matter of some doubt. It is not impossible that synthesis of hippuric acid takes place to a considerable extent in the kidney in accordance with the ideas of Bunge and Schmiedeberg (21). This hypothesis

is, however, doubted by Kingsbury and Bell (22). The conclusions of the latter may, however, be questioned since the assumption was made by them that the conjugated benzoic acid which the dog excretes is wholly in the form of hippuric acid. The increase in the total urinary nitrogen output following the ingestion of benzoic acid is probably due to a breakdown of body protein due to the toxicity of the benzoic acid.

The evidence which has thus far been presented seemingly

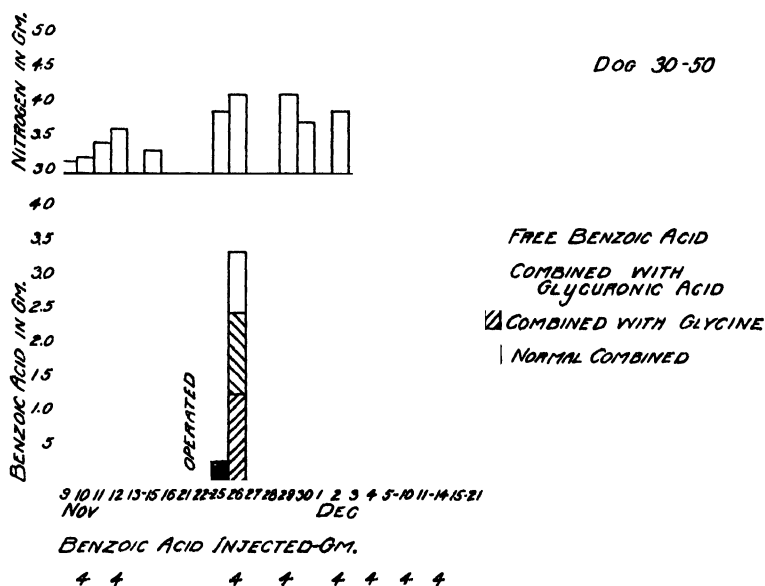


CHART XV.

points to the fact that as a result of jaundice impairment of liver function takes place. It could be expected that long continued biliary stasis should lead to morphological changes in the liver. While our experiments seemingly indicate functional disturbances in the activities of the liver soon after the onset of jaundice it seems probable that morphological changes cannot be detected as early as impairment of function. The histological studies were therefore begun some time after the onset of biliary stasis.

No work on this subject appears to have been carried out on the dog. In the human such studies have been made by Schwarz (23) and Eppinger (24).

The results recorded here were obtained from a study of the livers of two dogs, one 4 weeks and the other 11 weeks after the onset of jaundice. Sections which were made from the liver of the first animal showed a slight distortion of the trabeculae and a swollen condition of the bile ducts. The liver of the second

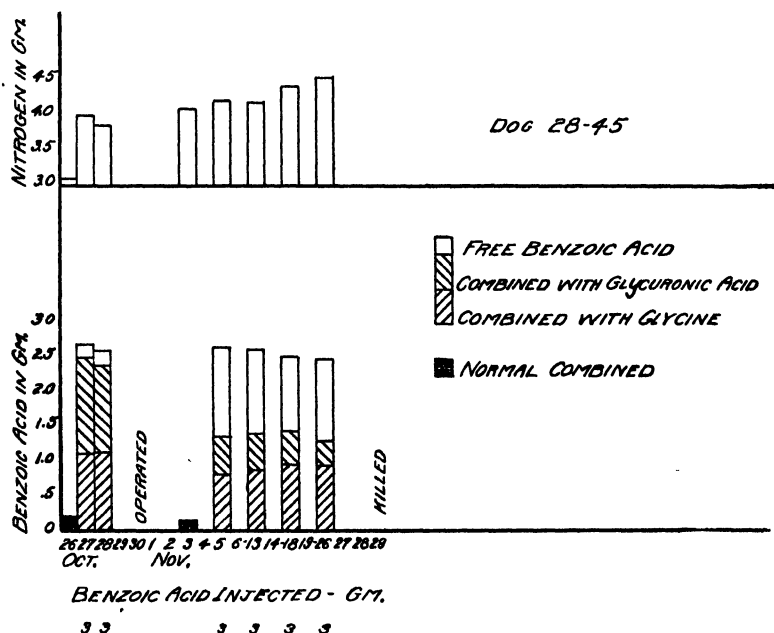


CHART XVI.

animal showed (a) the trabeculae considerably distorted and many of the cells broken down, (b) considerable increase over the normal amount of interlobular connective tissue, (c) large biliary thrombi around which were broad spaces where formerly blood capillaries were situated, (d) a large number of giant cells. Although no such studies were carried out it would be interesting to have histological data on other organs which were obtained under the same conditions.

Our experiments furnish no exact data on the question as to whether such amounts of bile acid which the jaundiced dog synthesizes are in large part broken down or stored in the body. The increase in urinary neutral sulfur following the onset of jaundice might suggest a hydrolytic cleavage of taurocholic acid. If this method is used by the body for the disposal of taurocholic acid it would not be expected that injection of bile acid at a time when the urinary output of this substance was at a low level

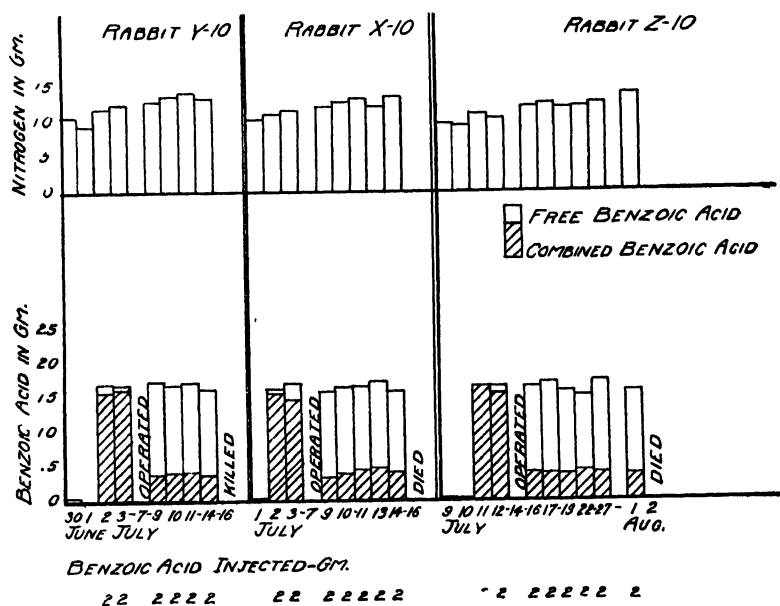


CHART XVII.

CHART XVIII.

CHART XIX.

would lead to an increased urinary output. On the other hand it is possible that the increased output of nitrogen and sulfur is due to extensive tissue destruction. It is of interest to note that the increased output of neutral sulfur in severe cases of jaundice has been observed by others (25). The loss in weight of the animals and the marked toxemia support this view. The decreased ability of the jaundiced animal to conjugate benzoic acid and the morphological changes which the liver undergoes after prolonged biliary stasis suggest that the decreased output of



taurocholic acid some time after the onset of jaundice is due to a decreased synthesis rather than breakdown of this substance.

#### SUMMARY.

1. Experiments were carried out to determine the fate of the bile acid and the bile pigments in jaundiced dogs. The jaundice was produced by ligation and subsequent resection of the common bile duct.

2. Daily estimation of nitrogen, total and inorganic sulfur, bile acid, and bile pigments was carried out on a series of dogs maintained on diets varying from a low to a high protein diet.

3. Following the onset of jaundice the output of nitrogen and sulfur increased and after an interval of a week or more gradually approached the normal level. Towards the end of the experiments the output of nitrogen again increased. The increase in the output of sulfur was largely confined to the neutral sulfur fraction.

4. The production of jaundice was followed by urinary excretion of bile acid and bile pigment. The output of bile acid decreased with time and finally became a very small quantity. Injection of taurocholic acid led to an increased excretion of this substance. The amount of bile pigments which was excreted became less some time after the onset of jaundice. A low but fairly constant excretory level was maintained.

5. The ability of the jaundiced dog and rabbit to detoxicate benzoic acid is markedly diminished after the onset of jaundice. In the dog this is chiefly shown by the decreased output of benzoylglycuronic acid.

6. Microscopic sections of the livers of two jaundiced dogs showed extensive morphological changes.

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## NOTE ON THE WYSS CHEMICAL METHOD OF ASSAYING INSULIN.

By FRITZ BISCHOFF, L. C. MAXWELL, AND N. R. BLATHERWICK.

*(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.)*

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In the Wyss<sup>1</sup> chemical method for the assay of insulin, it is assumed that the reaction is specific for the active insulin group, whatever that may be. Work in this laboratory does not affirm this assumption and proves rather conclusively that the reaction is general for proteins and even for amino acids like tyrosine and tryptophane. The principle of Wyss' method is as follows: in the presence of insulin the oxidation of the polyhydroxy phenols by hydrogen peroxide is inhibited. Advantage is taken of the power that insulin has of destroying hydrogen peroxide. The polyhydroxy phenols give rise to colored solutions when oxidized by peroxide. By running simultaneously a series of tests in which the same amounts of phenol and insulin, and varying but known amounts of peroxide are used, it is possible to find the point at which the insulin has destroyed all of the peroxide by the absence of any development of color. In trying out the test on some particular batch of insulin we found the line of demarcation between the colored and non-colored solutions to be very sharp and the results could be easily duplicated. Another batch of insulin, however, having the same potency (established by the rabbit method), but having a different nitrogen content, gave entirely different results. Three samples of insulin were used in our tests, containing 0.003, 0.016, and 0.070 mg. of nitrogen per rabbit unit. Contrary to expectations, the very pure insulin had the least effect in neutralizing the peroxide, while the impure sample had the greatest effect. The results showed that the number of units of insulin equivalent to a given

<sup>1</sup> Wyss, F., *Compt. rend. Acad.*, 1925, clxxxi, 327.

volume of Wyss' peroxide was inversely proportional (within the experimental error) to the nitrogen content per unit. This indicated that the test was not specific for insulin but depended rather upon the protein content. Casein and gelatin were now substituted for insulin. Casein solutions were found to have exactly the same power of destroying peroxide solutions as insulin solutions having the same nitrogen content. Gelatin had only one-fifteenth the power. Since gelatin contains no tyrosine or tryptophane and insulin<sup>2</sup> and casein contain both these amino acids, the Wyss test was also applied to these two amino acids. On an equal nitrogen basis it was found that tyrosine has about three times the power of destroying peroxide that either insulin or casein has, and tryptophane about one and a half times the power. In carrying out these assays, special attention was given to adjusting the pH at 7.5 as Wyss directs. Though troublesome, it is absolutely essential for consistent results.

*Summary of Results.*

Substance.	Weight required to neutralize 0.8 cc. Wyss $H_2O_2$ solution.	Weight of N in amount required.	Rabbit units represented.
	mg.	mg.	
Insulin B 269.....	0.28	0.040	2.5
“ A.....	0.24	0.035	0.5
“ purified.....	0.28	0.040	12.0
Casein.....	0.21	0.033	
Gelatin.....	3.06	0.480	
Tyrosine.....	0.16	0.013	
Tryptophane.....	0.17	0.024	

CONCLUSIONS.

The Wyss colorimetric method for the assay of insulin is not specific for insulin but applies also to proteins, such as casein, and to amino acids, such as tyrosine.

Tyrosine and tryptophane were found to be more sensitive to the test than either insulin or casein, but the power of these amino acids to destroy peroxide is not sufficient to ascribe the action of the insulin or casein entirely to their presence.

<sup>2</sup> Scott, D. A., *J. Biol. Chem.*, 1925, lxx, 601.

# THE INFLUENCE OF ASPHYXIATION ON THE BLOOD CONSTITUENTS OF MARINE FISHES.

BY F. G. HALL, I. E. GRAY, AND S. LEPKOVSKY.

(From the Zoological Laboratory of Milton College, Milton, and the University  
of Wisconsin, Madison.)

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## INTRODUCTION.

Changes in the constituents of the blood of fishes during asphyxiation have been noted by several authors. In a biochemical study of the blood of marine fish, Denis (1922) showed that a considerable variation occurs among individual fishes of the same species if they are kept in laboratory tanks. McCormick and Macleod (1925) found it necessary to keep fishes free from asphyxial conditions in their investigation of the effect of the removal of the principle islets. They also report a hyperglycemia following asphyxiation. They also made tests to show that increase of sugar is due to the presence of masked carbohydrates in the blood. Hemoglobin was the only other blood constituent studied by them. The senior author of this paper found a wide variation in the amount of hemoglobin in the blood of certain marine fishes if they were kept in laboratory tanks. This was especially true if the oxygen tension became low.

While important information is found in other investigations no very complete analysis has been made on a similar sample of fish blood for several organic and inorganic constituents during asphyxiation. For these reasons it was thought important to study the changes that might occur in the blood under different degrees of asphyxiation and to make as complete an analysis of the constituents as possible. The analyses were made for hemoglobin, iron, number of red blood corpuscles, percentage of formed elements, sugar, non-protein nitrogen, total nitrogen, chlorine, phosphorus, and percentage of dry matter. Only those results obtained from a study of the menhaden, *Brevoortia tyrannus*

(Latrobe), are reported here although other species were used. Results obtained on the puffer, sea-robin, and scup will be reported in another paper.

This investigation was carried on in the laboratory of the United States Bureau of Fisheries at Wood's Hole, Massachusetts. The authors are greatly indebted to the Bureau for assistance, especially to Dr. Willis R. Rich for valuable criticisms and suggestions. The authors also wish to express thanks to Dr. H. C. Bradley and Dr. A. S. Pearse for their interest and helpful suggestions.

### *Methods.*

Fishes used in this experiment were caught in traps such as those employed by the Atlantic fishermen. The traps were visited in the morning and the fish brought to the laboratory where they were kept in a large floating "live car." It was found that after living in the car from 24 to 48 hours that the blood constituents showed a minimum variation.

To obtain different degrees of asphyxiation fishes were placed separately in museum jars containing approximately 10 liters of water. The jars were immersed in a small hatchery box, one jar in each box. Water was kept running through the boxes. The jars were sealed with glass stoppers. Thus the temperature was kept constant and the exact degree of asphyxiation determined for each fish. The Winkler method for the determination of dissolved oxygen as modified by Birge and Juday (1911) was used. Determination of the oxygen was made just before the fishes were put in the jars. At 10 minute intervals fishes were removed and a sample of the water was taken for oxygen determination. Each fish was quickly bled by cutting off the tail and the blood caught in an oxalated Erlenmeyer flask. Care was taken to prevent tissue fluids and lymph from entering the flask. Lithium oxalate was used as an anticoagulant. The chemical analyses were made on whole blood. The temperature was maintained between 19–20°C.

*Hemoglobin.*—Hemoglobin was determined by the Newcomer method. A biological colorimeter fitted with a Newcomer standard for acid hematin was used. The determinations are expressed as percentages based on the Williamson standard, in which 100 per cent is equivalent to 16.92 gm. of hemoglobin per 100 cc.

**Corpuscles.**—The red blood corpuscles were enumerated by the standard clinical method. A counting slide with a Neubauer ruling was used. The results are expressed as number of corpuscles per c.mm.

**Percentage of Formed Elements.**—About 10 cc. of the whole blood (oxalated) were centrifuged in a graduated tube for 30 minutes at about 2800 R.P.M. The percentage of corpuscles in the blood was then determined.

**Iron.**—Iron was determined by the Wong method. It was found that if the sulfuric acid was diluted to about two-thirds strength instead of the concentration given by Wong (1923) the color would not fade as rapidly and that more accurate readings could be made.

**Sugar, Non-Protein Nitrogen, Chlorine.**—For these constituents Folin's methods were used as described in his manual (1923). A non-protein filtrate was prepared and the three constituents determined on samples of the same filtrate.

TABLE I.

*Average Results of Analyses of Blood Constituents of Normal Fish and of Fish at Different Degrees of Asphyxiation.*

Time of asphyxiation.	Hemoglobin.	Iron.	Non-protein nitrogen.	Total nitrogen.	Phosphorus.	Chlorine.	Dry matter.	Corpuscles.	Red blood cells, per c.mm.	Dissolved oxygen.
min.	per cent	mg. per 100 cc.	mg. per 100 cc.	mg. per cc.	mg. per 100 cc.	mg. per cc.	per cent	per cent		cc. per l.
Normal.	53.3	32.9	41.3	24.2	11.1	5.23	12.10	29.5	1,988,000	4.87
10	63.5	43.0	44.6	24.6	13.8	5.37	16.18	45.3	2,718,000	2.30
20	63.1	45.3	46.4	25.9	14.9	5.22	16.35	45.1	2,366,000	0.93
30	70.8	50.5	51.1	30.2	16.8	5.18	17.28	47.9	2,603,000	0.63
40	85.2	67.0	57.6	31.5	17.7	5.22	20.64	62.6	3,229,000	0.43
50	92.0	67.2	59.8	32.3	18.8	5.08	20.72	68.9	3,598,000	0.29

**Total Nitrogen.**—Total nitrogen was determined as directed by Folin's manual and is expressed as mg. per cc.

**Dry Matter.**—About 2 cc. of whole blood were weighed and dried at 95°C. until the weight became constant. The ratio of dry weight to the initial weight was thus determined.

### Results.

72 menhaden were used in the experiments. The blood of one normal fish and one asphyxiated for each 10, 20, 30, 40, and 50 minute period was analyzed each day. The results are therefore comparable. In Table I averages of the individual analyses are given except in the case of sugar. These analyses show that the constituents of the blood exclusive of sugar and chlorine become



increasingly concentrated as the asphyxiation of the fish progresses. The decrease in the oxygen tension during asphyxiation is shown in Table I.

The concentration of sugar varied considerably but showed no progressive variation in concentration during asphyxiation. In Table II are shown the results of analysis for individual fishes during asphyxiation.

The chlorine concentration showed little change during asphyxiation.

TABLE II.

*Mg. of Sugar per 100 Cc. of Blood of Normal Menhaden and of Menhaden during Different Degrees of Asphyxiation.*

Normal.	Minutes.				
	10	20	30	40	50
99.3	85.7	72.4	98.0	75.0	99.3
87.7	317.4	185.1	170.9	151.5	294.1
215.0	63.2	99.5	42.6	93.9	183.4
75.0	104.7	210.5	143.8	50.0	76.9
82.3	136.0	215.0	180.1	80.0	105.8
94.3	138.8	158.7	215.0	200.0	192.3
Average....108.9	140.9	156.9	141.7	108.4	158.6

## DISCUSSION.

The results obtained in this investigation indicate that asphyxiation brings about an increased concentration of many of the blood constituents. With the exception of chlorides and sugars the increased concentration is apparently gradual and reaches its highest concentration just before the death of the fish. This indicates that the concentration is mainly caused by the release of water from the blood and not an actual increase of each of these constituents. In other words the blood has apparently become more concentrated by a reduction in blood volume, which may thus become reduced to nearly one-half the normal.

These results do not show the destination of the water. It is suggested that the water passes out of the blood into the tissues. During asphyxiation the tissues very likely become acid. An increased acidity would bring about an edema and the tissues would absorb water from the blood. The longer and greater the

asphyxiation, the more water would be absorbed and the more concentrated the blood would become. It is known that acids retard tissue respiration, thus there is set up in the tissues a vicious cycle:—asphyxiation—more acid, more acid—greater asphyxiation, etc.

Because of the uniformity in the concentration of chlorine in the blood during asphyxiation it would seem that chlorine passes out of the blood simultaneously with water. It probably passes along with water from the blood to the tissues and thereby maintains their osmotic pressure constant. This process may be considered adaptive.

McCormick and Macleod found an increase in blood sugar in fish during asphyxiation. We did not find this to be the case with menhaden. The individual variation was greater than that caused by asphyxiation indicating that other factors may cause wider variation than does asphyxiation. One may conclude from our work that a study of a single substance in blood of fishes during asphyxiation has little value if the degree of asphyxiation is not known. Reports stating that some substance increases or decreases is not significant unless the physiological state of the animal is known.

The results presented in this paper shed some light on the processes involved in asphyxiation in fishes. Perhaps the death of a fish from asphyxiation is due in part to the retention of substances which are toxic and which during asphyxiation become concentrated through loss of water from the blood.

#### SUMMARY.

1. In the menhaden asphyxiation produces a definite increased concentration of the following blood constituents: hemoglobin, phosphorus, iron, non-protein nitrogen, total nitrogen, number of red blood corpuscles, percentage of formed elements, and percentage of dry matter. The increase is roughly proportional to the length of time of asphyxiation.

2. Blood sugar is apparently influenced to a greater extent by other factors than by asphyxiation.

3. The chloride concentration is maintained nearly constant during asphyxiation.

4. The increased concentration of the various blood constituents can be explained by the release of water from the blood. Thus the blood volume apparently becomes diminished during asphyxiation.

5. It is suggested that increased tissue acidity during asphyxiation brings about the transfer of water from the blood to the tissues, and that death may perhaps be due to the retention or concentration of toxic substances.

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## COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

### IX. FATE OF SOME HALOGEN DERIVATIVES OF BENZOIC ACID IN THE ANIMAL BODY.

BY N. JEAN NOVELLO, S. R. MIRIAM, AND CARL P. SHERWIN.

(From the Chemical Research Laboratory of Fordham University, New York.)

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In the literature one finds comparatively few references to the fate of the benzoic acid derivatives in the animal body, and even some rather conflicting statements regarding several of the corresponding hippuric acids. The object of our work, therefore, was to prepare the *o*-, *m*-, and *p*-chloro-, bromo-, and iodobenzoic acids, and, by feeding these substances to dogs and rabbits, to compare the products isolated from the urine with the corresponding hippuric acids synthesized in the laboratory.

Preusse (1) fed *p*-bromotoluene to dogs and recovered a substance from the urine which he believed was *p*-bromohippuric acid. Coppola (2) found that *o*-, *m*-, and *p*-fluorobenzoic acids when fed to dogs were converted into the respective fluorohippuric acids. Graebe and Schultzen (3) found that after the ingestion of *m*-chlorobenzoic acid a new compound appeared in the urine which they believed to be *m*-chlorohippuric acid. Though they were unable to isolate this new substance in crystalline form they succeeded in preparing its calcium salt, which on analysis yielded results comparable to those for calcium chlorohippurate. Hildebrandt (4), experimenting with dogs, fed the *o*-, *m*-, and *p*-chlorotoluenes, *o*-bromotoluene, and *m*-bromobenzoic acid. From the *o*-chlorotoluene he obtained *o*-chlorobenzoic acid, together with an impure substance which he believed was *o*-chlorohippuric acid. The calcium salt of this latter compound gave analytical results corresponding with those for calcium *o*-chlorohippurate. The *m*-chlorotoluene gave a syrupy material which yielded on hydrolysis with hydrochloric acid *m*-chlorobenzoic acid. The feeding of *p*-chlorotoluene was followed by the appearance in the urine of *p*-chlorohippuric acid (m.p. 143°C.). After feeding *o*-bromotoluene he found *o*-bromohippuric acid in the urine (m.p. 153°C.); and after the administration of *m*-bromo-

benzoic acid he recovered what seemed to be *m*-bromohippuric acid (m.p. 183°C.), for on hydrolysis with concentrated hydrochloric acid it produced some *m*-bromobenzoic acid. He also fed the *o*-, *m*-, and *p*-chlorotoluenes, and the *o*-, *m*-, and *p*-bromotoluenes to rabbits. From the chloro compounds he recovered only the respective benzoic acids. The bromo derivatives, however, as a rule went further; thus, the *o*-bromotoluene was converted almost quantitatively into *o*-bromohippuric acid; *m*-bromotoluene was excreted partly as *m*-bromobenzoic acid and partly as *m*-bromohippuric acid; while *p*-bromotoluene was eliminated largely as *p*-bromohippuric acid, only a small portion of it appearing as *p*-bromobenzoic acid.

#### EXPERIMENTAL.

The substances were fed *via stomach tube* to both dogs and rabbits as water solutions of the respective sodium salts. The individual doses, depending on the toxicity of the substances as well as on the size of the animal, ranged from 0.5 to 2 gm. for the rabbit and from 2 to 5 gm. for the dog. One dose was given every 24 hours until a total of at least 5 gm. had been fed. The animal was then kept in the metabolism cage until the 36th hour after the last feeding. The urine, after being carefully and accurately neutralized, was evaporated on the water bath to a thick syrup, cooled, acidified with sulfuric acid to Congo red, and extracted in a rotary extracting apparatus with some suitable solvent such as ether, ethyl acetate, or alcohol. The extract was then evaporated almost to dryness, taken up with hot water, and decolorized with charcoal. The substance was then allowed to crystallize out, after which it was recrystallized several times from hot water and dried *in vacuo*. Melting point determination and analysis followed.

#### *o*-Chlorobenzoic Acid.

A rabbit was fed 3 gm. of *o*-chlorobenzoic acid on each of 3 consecutive days. Upon extracting with ethyl acetate, 2.24 gm. of the free substance were recovered from the urine.

A dog was given the same substance in two doses of 3 gm. each. Of the unchanged substance 4.1 gm. were extracted from the urine.

In neither case was there any apparent conjugation with glycocholl.

*Synthesis of o-Chlorohippuric Acid.*

This substance was prepared by the action of *o*-chlorobenzoyl chloride on glycocoll according to the Schotten and Baumann reaction. The *o*-chlorobenzoyl chloride was prepared as follows: 5 gm. of *o*-chlorobenzoic acid (m.p. 137°C.) were treated under a reflux on a water bath with ten times its weight of thionyl chloride until the benzoic acid went into solution. The resulting *o*-chlorobenzoyl chloride was then distilled off under reduced pressure. Boiling point at 9 mm. pressure, 87°C.; yield, 87 per cent theoretical.

10 gm. of *o*-chlorobenzoyl chloride were gradually added with constant shaking to 3.5 gm. of glycocoll dissolved in water. Sodium hydroxide was added at intervals in quantity just sufficient to keep the reacting mixture neutral or slightly alkaline. After the reaction was completed the entire mass was acidified strongly with sulfuric acid. The precipitate was then filtered by suction, washed repeatedly with ice water, and extracted with petroleum ether, thus removing the free *o*-chlorobenzoyl chloride as well as the *o*-chlorobenzoic acid. The *o*-chlorohippuric acid was then recrystallized several times from hot water and dried *in vacuo*, after which it showed a constant melting point of 176°C. The substance has a light yellow color and crystallizes from hot water in thin leaves. It is easily soluble in hot water, alcohol, and ethyl acetate, but insoluble in petroleum ether, benzene, ether, and chloroform. The following analytical results were obtained. Kjeldahl nitrogen: found, 6.51 and 6.57 per cent; calculated, 6.55 per cent. Per cent chlorine according to the method of Carius: found, 16.79 and 16.88 per cent; calculated, 16.62 per cent. The calcium and barium salts form insoluble amorphous substances. The copper salt is quite soluble and crystallizes from hot water in green platelets.

*m*-Chlorobenzoic Acid.

A rabbit received 3 gm. of *m*-chlorobenzoic acid. About 2 gm. of the free substance were recovered from the urine. Ethyl acetate was employed as the solvent.

A dog was fed 8 gm. of *m*-chlorobenzoic acid in two doses of 4 gm. each. From the urine 6 gm. of the free substance were recovered.

In neither case was there evidence of a conjugation with glycocoll.

*Synthesis of m-Chlorohippuric Acid.*

20 gm. of *m*-chlorobenzoic acid were treated in the usual way with 27 gm. of phosphorus pentachloride. This yielded 17 gm. of *m*-chlorobenzoyl chloride, which boiled at 110°C. under a pressure of 15 mm.

This 17 gm. of *m*-chlorobenzoyl chloride was then treated with 5 gm. of glycocoll according to the Schotten and Baumann method. A yield of 10 gm. of *m*-chlorohippuric acid was obtained, which after careful drying *in vacuo* melted at 143–144°C. The substance crystallizes from water in clusters of long, thin needles. It is quite easily soluble in hot water and alcohol, and very soluble in pyridine, but practically insoluble in cold water, ether, petroleum ether, and benzene. Nitrogen analysis according to Kjeldahl method resulted as follows: found, 6.60 and 6.65 per cent; calculated for  $C_9H_8NO_3Cl$ , 6.56 per cent. Chlorine determination after the method of Carius gave the following figures: found, 16.14 and 16.56 per cent; calculated, 16.67 per cent.

*p*-Chlorobenzoic Acid.

After three 1 gm. doses of this substance were fed to a rabbit, 2.2 gm. of the unchanged material were recovered from the urine. No *p*-chlorohippuric acid could be found.

Two 5 gm. doses of the same substance were fed to a dog. After extracting the urine with ether we recovered 3.3 gm. of free *p*-chlorobenzoic acid (m.p. 231–235°C.), together with small amounts of a substance with a lower melting point. After extracting the evaporated urine with ethyl acetate we recovered a much larger amount of this latter substance, which we were able to purify by fractional crystallization from ether. This substance then melted at 141–143°C., and was recognized by its nitrogen content as *p*-chlorohippuric acid, for the nitrogen determination according to Kjeldahl showed 6.61 and 6.78 per cent. The theoretical percentage is 6.56. The total amount of the substance isolated from the dog's urine after feeding the 10 gm. of *p*-chlorobenzoic acid was 4.1 gm.

*Synthesis of p-Chlorohippuric Acid.*

This synthesis was effected in the usual way from *p*-chlorobenzoyl chloride and glycocoll. The *p*-chlorobenzoyl chloride was obtained from *p*-chlorobenzoic acid by means of phosphorus pentachloride. Thus 4 gm. of *p*-chlorobenzoyl chloride were prepared from 10 gm. of *p*-chlorobenzoic acid. The product showed a boiling point of 210–212°C. at 16 mm. pressure, with the bath at a temperature of about 250°C.

This 4 gm. of *p*-chlorobenzoyl chloride was treated with 3 gm. of glycocoll according to the method of Schotten and Baumann. After the completion of the reaction the mixture was acidified with sulfuric acid and then extracted repeatedly with ethyl acetate. This removed both the *p*-chlorobenzoic acid and the *p*-chlorohippuric acid. This extract was then evaporated, after which the residue was repeatedly extracted with petroleum ether, which removed the uncombined *p*-chlorobenzoic acid. The *p*-chlorohippuric acid was then purified by several recrystallizations from water. Yield, 2.3 gm.; melting point, 143°C. The substance is difficultly soluble in cold water, ether, petroleum ether, and carbon tetrachloride, but easily soluble in alcohol, ethyl acetate, and hot water. It crystallizes in the form of white, irregular leaflets. Kjeldahl nitrogen analysis: found, 6.86 and 6.36 per cent; calculated for  $C_9H_8O_3NCl$ , 6.56 per cent. Carius chlorine analysis: found, 16.81 and 16.77 per cent; calculated, 16.67 per cent. The properties of this synthetic compound agree with those of the product isolated from the urine of a dog by Hildebrandt (4) after the feeding of *p*-chlorotoluene, and by us after the administration of *p*-chlorobenzoic acid.

*o-Bromobenzoic Acid.*

A total of 6 gm. of *o*-bromobenzoic acid was fed to a dog in two 3 gm. doses. From the urine 5 gm. of *o*-bromohippuric acid were isolated.

Two 1 gm. doses of *o*-bromobenzoic acid were given to a rabbit, and about 2 gm. of *o*-bromohippuric acid were obtained from the urine. This product, after recrystallization, melted at 185–190°C. Kjeldahl nitrogen analysis indicated 5.56 and 5.61 per cent, instead of the theoretical percentage of 5.42.



*Synthesis of o-Bromohippuric Acid.*

*o*-Bromobenzoyl chloride was first prepared by the interaction of 30 gm. of *o*-bromobenzoic acid and 31 gm. of phosphorus pentachloride in the usual way. Thus, 25 gm. of *o*-bromobenzoyl chloride were obtained by distillation at 118°C. and 10 mm. pressure. This 25 gm. of *o*-bromobenzoyl chloride was then gradually added with continuous shaking to a water solution of 5.8 gm. of glycocoll. The reacting mixture was kept faintly alkaline by the occasional addition of a few drops of dilute sodium hydroxide solution. After the reaction was finished the mixture was acidified with sulfuric acid and extracted repeatedly with petroleum ether to remove the uncombined *o*-bromobenzoic acid. The *o*-bromohippuric acid was then separated by filtration and recrystallized several times from hot water. After drying *in vacuo* it melted at 192–193°C. It is a white substance, crystallizes in the form of short, thick needles, is insoluble in cold water, butyl alcohol, ether, and petroleum ether, but is very soluble in hot water and ethyl acetate. Nitrogen determination according to Kjeldahl method gave 5.48 and 5.51 per cent; calculated for  $C_9H_8O_2NBr$ , 5.42 per cent. Percentage bromine: found, 31.56 and 31.42 per cent; calculated, 31.00 per cent.

*m*-Bromobenzoic Acid.

Three 1 gm. doses of this substance were fed to a rabbit. Only the uncombined substance could be recovered from the urine to the amount of 2.12 gm.

After feeding 8 gm. of *m*-bromobenzoic acid to a dog in two doses, we isolated from the urine a mixture containing 3.3 gm. of *m*-bromobenzoic acid (m.p. 153–156°C.) and 2.8 gm. of *m*-bromohippuric acid (m.p. 148–150°C.). This *m*-bromohippuric acid did not agree in melting point with the substance which Hildebrandt (4) isolated from a dog's urine after the feeding of *m*-bromobenzoic acid, and which he believed was *m*-bromohippuric acid. The substance isolated by us gave nitrogen percentages, as determined by the Kjeldahl method, of 5.30 per cent and 5.26 per cent. The theoretical value is 5.42 per cent.

*Synthesis of m-Bromohippuric Acid.*

The synthesis was carried out as in the cases previously described; that is, by the action of *m*-bromobenzoyl chloride on glycocoll according to the Schotten and Baumann reaction. 2 gm. of *m*-bromobenzoic acid (m.p. 155°C.) were refluxed on the water bath with 10 gm. of thionyl chloride until the acid had completely gone into solution. After the excess thionyl chloride had been removed by suction, the *m*-bromobenzoyl chloride was distilled off at 122.5–123.5°C. and 14 to 15 mm. pressure. Yield, 90 per cent of the theoretical. Analysis for chlorine showed the figures of 13.21 and 13.25 per cent, instead of the theoretical amount of 13.00 per cent.

6½ gm. of *m*-bromobenzoyl chloride were gradually added, with constant shaking, to a water solution of 2.5 gm. of glycocoll. Alkali was added now and then to maintain neutrality. After the reaction was completed the solution was acidified to Congo red with sulfuric acid. The precipitate was first extracted with benzene to remove the free *m*-bromobenzoic acid. The *m*-bromohippuric acid was then filtered off and repeatedly recrystallized from hot water. It separates out in the form of clusters of long needles. It is soluble in hot water, methyl alcohol, ethyl alcohol, and glacial acetic acid. It is sparingly soluble in toluene, benzene, ether, and cold water. Dried *in vacuo* it melts at 146–147°C. Nitrogen analysis according to the method of Kjeldahl gave the figures of 5.37 and 5.32 per cent, instead of the theoretical value of 5.42 per cent. Chlorine analysis (Carius) showed 31.30 and 31.19 per cent, as compared with the calculated percentage of 31.01.

*m-Bromohippuric Cyanide (-Hippuronitrile).*

15 gm. of amino acetonitrile sulfate were dissolved in 15 cc. of water. To this were added with constant shaking 7.3 gm. of *m*-bromobenzoyl chloride dissolved in 25 cc. of benzene. The reacting mixture was kept faintly alkaline by the occasional addition of a small amount of very dilute sodium hydroxide. Yield, 4.45 gm., or 75 per cent theoretical; melting point, 103.5–104.5°C. The substance is very soluble in methyl alcohol, ethyl alcohol, and glacial acetic acid; it is moderately soluble in hot water and

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ether, but insoluble in cold water, benzene, and toluene. Carius bromine determination showed 33.47 per cent instead of the theoretical 33.41 per cent.

### *Ethyl m-Bromohippurate.*

1 gm. of *m*-bromohippuryl cyanide was dissolved in 10 cc. of absolute alcohol, saturated with dry hydrogen chloride (gas), and heated on a water bath for 3 minutes. The product settles out in the form of an oil. B.P. 110–118°C.

### *Silver m-Bromohippurate.*

1 gm. of *m*-bromohippuric acid was dissolved in ammonium hydroxide. The solution was then neutralized with nitric acid, and the silver salt was precipitated with silver nitrate. The substance precipitates as an amorphous mass. Analysis showed the presence of 28.71 per cent silver, instead of the theoretical amount of 29.39 per cent.

### *p-Bromobenzoic Acid.*

This substance was fed to rabbits in doses of 1 gm. until a total of 5 gm. had been given, and to dogs in doses of 3 gm. until a total of 12 gm. had been administered. It was largely excreted as *p*-bromohippuric acid, with only traces of the free *p*-bromobenzoic acid. Thus, 63 per cent of the substance fed to rabbits, and 72 per cent of that fed to dogs, was recovered in the urine in the form of *p*-bromohippuric acid. From hot water the material crystallized in clusters of long, flat needles. When dried *in vacuo* it melted at 159–162°C., thus agreeing with the synthetic product prepared by Klages and Haack (5).

### *o-Iodobenzoic Acid.*

This substance was fed to a rabbit in doses of 1 gm. on each of 3 consecutive days. From the urine 1.8 gm. of the unchanged material (m.p. 161–162°C.) were recovered.

The feeding of two doses of 3 gm. each of the same compound to a dog resulted in the excretion of 1 gm. of the unchanged substance and of 2.5 gm. of *o*-iodohippuric acid (m.p. 167–170°C.).

This latter material, when analyzed for nitrogen by the Kjeldahl method, showed the values of 4.38 and 4.49 per cent, instead of the theoretical amount of 4.59 per cent.

*Synthesis of o-Iodohippuric Acid.*

*o*-Iodobenzoyl chloride was first prepared in the usual way; that is, by treating 25 gm. of *o*-iodobenzoic acid (m.p. 161–162°C.) with 21 gm. of phosphorus pentachloride and 40 gm. of phosphorus oxychloride. After the completion of the reaction the mixture was extracted with absolute ether. Thus, 25 gm. of *o*-iodobenzoyl chloride (m.p. 40°C.) were obtained. This material was then added, following the usual technique, to 5 gm. of glyco-coll dissolved in water. After the completion of the reaction the contents of the flask were acidified strongly with sulfuric acid and then extracted repeatedly with benzene to remove the uncombined *o*-iodobenzoic acid. The *o*-iodohippuric acid was then taken up in hot water, from which it crystallized in the form of long, slender, white needles. Melting point, 170°C. It is soluble in hot water, ether, alcohol, ethyl acetate, and chloroform, but very slightly soluble in cold water and benzene. The following analyses were obtained after the substance had been carefully dried *in vacuo*. Nitrogen (Kjeldahl): found, 4.42 and 4.49 per cent; calculated, 4.58 per cent. Carbon (combustion): found, 35.32 and 35.26 per cent; calculated, 35.41 per cent. Hydrogen (combustion): found, 3.01 and 2.87 per cent; calculated, 2.62 per cent. Titration, 0.2527 gm. substance required 8.10 cc. 0.1 N NaOH; calculated, 8.28 cc. 0.1 N NaOH.

*m*-Iodobenzoic Acid.

This compound was fed to a rabbit in doses of 1 gm. per day until a total of 5 gm. had been given. Only uncombined *m*-iodobenzoic acid could be found in the urine, and of this only about 2 gm. (m.p. 183–187°C.).

After feeding 9 gm. of *m*-iodobenzoic acid to a dog in doses of 3 gm. each, we recovered from the urine 3.1 gm. of the unchanged acid, together with a syrupy mass which was very soluble in ethyl acetate and only sparingly soluble in ether. This mass, when boiled with concentrated hydrochloric acid, yielded both

*m*-iodobenzoic acid and iodine. The calcium salt was prepared by dissolving the syrupy material in calcium hydroxide and then removing the excess calcium with carbon dioxide. This calcium salt precipitated as a rather insoluble, amorphous product with an indefinite melting point. Kjeldahl nitrogen determination showed the figures of 2.01 and 2.06 per cent, whereas the theoretical value for  $\text{Ca}(\text{C}_6\text{H}_4\text{INO}_2)_2$  is 2.31 per cent.

*Synthesis of m-Iodohippuric Acid.*

This synthesis was effected by much the same procedure as we employed in the above mentioned cases; that is, by first preparing *m*-iodobenzoyl chloride by the action of thionyl chloride on *m*-iodobenzoic acid, and then by treating the resulting product with glycocoll. Much difficulty was met with in this latter step, although we finally succeeded in obtaining the calcium salt of the desired substance. The free *m*-iodohippuric acid was then prepared from this calcium salt by suspending the latter in water in the proportion of about 1 gm. of the substance to approximately 200 cc. of water, and then by adding just enough sulfuric acid to remove the calcium. A tarry mass was thus formed, which, however, gradually dissolved as the water was boiled. The solution was then filtered and allowed to cool very slowly. The *m*-iodohippuric acid then crystallized out in the form of white leaflets, melting at 167–169°C. The compound is rather difficultly soluble even in hot water, and only slightly more easily soluble in alcohol and ether. It is quite insoluble in acetone, chloroform, carbon tetrachloride, and benzene. Kjeldahl nitrogen analysis showed 4.71 and 4.68 per cent, instead of the theoretical 4.59 per cent.

*p*-Iodobenzoic Acid.

This substance was fed to a rabbit in 1 gm. doses until 3 gm. had been given. After extracting the evaporated urine with ethyl acetate, we found a mixture of *p*-iodobenzoic acid and *p*-iodohippuric acid—0.8 gm. of the former and 1.2 gm. of the latter. The separation of the two compounds is quite easy on account of the greater solubility of the *p*-iodohippuric acid in warm water. The *p*-iodohippuric acid thus obtained melted at 184–186°C.

The melting point of the uncombined *p*-iodobenzoic acid was approximately 270°C.

After feeding a dog a dose of 3 gm. of *p*-iodobenzoic acid on 1 day and another dose of 4 gm. on the following day, we obtained 4.8 gm. of *p*-iodohippuric acid from the urine, but were unable to find any trace of uncombined *p*-iodobenzoic acid. The analysis of the *p*-iodohippuric acid for Kjeldahl nitrogen showed the presence of 4.76 and 4.69 per cent; the per cent calculated for  $C_9H_8NO_3I$  is 4.60.

#### *Synthesis of p-Iodohippuric Acid.*

This substance was prepared by the interaction of *p*-iodobenzoyl chloride (b.p. 126°C. at 9 mm. pressure) with glycocoll after the method of Schotten and Baumann. The resulting product melted at 188–190°C. It crystallizes from hot water in slightly yellow leaflets. It is difficultly soluble in hot water, though it dissolves fairly easily in alcohol and ethyl acetate. It is extremely insoluble in cold water, acetone, ether, and carbon tetrachloride. Nitrogen determinations (Kjeldahl) resulted as follows: found, 4.46 and 4.69 per cent; calculated for  $C_9H_8NO_3I$ , 4.59 per cent. Combustion gave the following figures: carbon, found, 41.40 and 41.49 per cent; calculated, 41.64 per cent; hydrogen, found, 3.01 and 2.90 per cent; calculated, 2.62 per cent.

#### SUMMARY.

The *o*-, *m*-, and *p*-chloro-, bromo-, and iodobenzoic acids were fed to dogs and rabbits. Although the dosage to rabbits was relatively much larger than that to dogs, there was less tendency on the part of the rabbit to join the substances with glycocoll.

Of the nine compounds fed, only three were joined with glycocoll by the rabbit; namely the *o*- and *p*-bromobenzoic acids and *p*-iodobenzoic acid.

On the contrary, the dog conjugated seven of the substances with glycocoll, leaving uncombined only the *o*- and *m*-chlorobenzoic acids.

The hippuric acids corresponding to these various monohalogen benzoic acids were prepared synthetically.

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## LIPASE STUDIES.

### III. THE HYDROLYSIS OF THE ESTERS OF THE DICARBOXYLIC ACIDS BY THE LIPASE OF THE LIVER.

BY DANIEL A. MCGINTY AND HOWARD B. LEWIS.

*(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor.)*

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In previous papers (1, 2), studies of the hydrolysis of the diethyl esters of malonic, succinic, glutaric, and adipic acids by lipase have been reported. On the basis of the acidity developed when a glycerol extract of hog liver was allowed to act on dilute solutions (0.025 and 0.05 N) of these esters, it was shown that in the hydrolysis of the diethyl esters of the two acids of lower molecular weight by liver lipase, equilibrium was reached when one ethyl group had been removed, and that further hydrolysis proceeded very slowly. With the esters of glutaric and adipic acids, on the other hand, the enzymatic hydrolysis proceeded rapidly until approximately 85 per cent of the ethyl groups present had been removed, the course of the reaction being similar to that observed in the hydrolysis of the esters of the monocarboxylic fatty acids of the aliphatic series (*e.g.* ethyl propionate) by lipase. No chemical differences in the properties of the esters or of the acids were known by which this marked difference in the hydrolysis of the esters by liver lipase could be explained.

It has seemed desirable to continue the study of the hydrolysis of the esters of the dicarboxylic acids by liver lipase and to investigate further the various factors concerned in the reaction. We have accordingly studied the action of liver lipase on monoethyl adipate, sodium ethyl adipate, diethyl maleate, diethyl fumarate, and diethyl *dl*-malate.

#### EXPERIMENTAL.

The methods were in general similar to those used in earlier studies (1, 2). Inasmuch as it was frequently difficult to weigh



out exactly the required amounts of the liquid esters, solutions of a normality approximating that desired were prepared and the exact value of these solutions in terms of the standard sodium hydroxide (0.1 N) required for complete saponification was determined. In order to insure complete solution of the esters, low concentrations (usually 0.025 N) were used. The lipase preparation was a strained glycerol extract of fresh hog liver prepared according to the procedure of Kanitz (3). This extract maintained its lipolytic activity unimpaired for several months.

25 cc. of the solutions of the esters were mixed with 0.5 cc. of the glycerol extract of the liver, the flasks were incubated at room temperature (20–23°) for varying periods of time, and the acidity developed in the reaction was then titrated with standard alkali, either 0.1 N sodium hydroxide or 0.0465 N calcium hydroxide, with phenolphthalein as an indicator. The flasks were arranged in pairs and one pair was titrated after 30 minutes (in a few cases after a shorter time interval), a second pair after 1 hour, etc. Each pair of the flasks was also retitrated at the intervals shown in the tables and the figures given represent the total volume of standard alkali required for neutralization in each case. The figures reported in the tables are the averages of these duplicate determinations, from which the blanks due to the acidity of the extract and the esters have been subtracted. In all the tables, the last figure in each vertical column represents the amount of alkali required for the neutralization of the acidity developed during the period indicated. Each figure in the horizontal column to the right of the first figure represents this amount of alkali plus the additional amounts of alkali required for retitration at the intervals indicated.

In order to check the results previously obtained, the course of the hydrolysis of diethyl malonate and diethyl adipate by the lipase preparation of the present series was investigated. The results (not included in the tables) were in complete agreement with our earlier studies. It was also considered possible that the results obtained on the retitrations of the flasks might in some way be influenced by the sodium ion introduced in the neutralization by sodium hydroxide, either by the ionization of the sodium salt of the monoethyl ester, if present, or by inactivation of the enzyme. In the case of each ester studied in the present series,

an experiment similar to that reported in the table, but differing in the fact that the neutralization of the acidity developed as a result of the enzymatic hydrolysis has been effected by calcium hydroxide, has been carried out. The course of the hydrolysis has been comparable in every way to that in those experiments in which sodium hydroxide was used to neutralize the acidity developed.

*Hydrogen Ethyl Adipate (Table I).*—In previous work (1) it was noted that acid ethyl malonate was not hydrolyzed by lipase of the liver, an observation which was in agreement with the theory

TABLE I.

*Hydrolysis of 0.025 N Hydrogen Ethyl Adipate by Lipase.*

25 cc. portions of the ester were used. For complete saponification of the ethyl group 3.25 cc. of 0.1000 N NaOH were required. In this table the blanks due to the initial acidity of the acid ester (3.16 cc. of 0.1000 N NaOH) have been deducted. The figures therefore represent alkali used to neutralize the acidity resulting from saponification of the ethyl group.

Time. ....15 min.	30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.
Standard NaOH required for neutralisation of acidity.						
cc. 1.14	cc. 1.14 1.82	cc. 1.14 1.82 2.53	cc. 1.23 1.88 2.58 2.85	cc. 1.23 1.88 2.58 2.85 2.90	cc. 1.23 1.88 2.58 2.85 2.90 2.90	cc. 1.23 1.88 2.58 2.85 2.90 2.90

that lipase removed one ethyl group of diethyl malonate readily, but that the second ethyl group was hydrolyzed with difficulty. Since diethyl adipate was found to be hydrolyzed to adipic acid with the removal of both ethyl groups, it might have been anticipated that hydrogen ethyl adipate would also be readily split by the enzyme. This prediction was verified by the results of the experimental work. At the end of the 2 hour period, 2.85 cc. of alkali were required to neutralize the acidity developed, equivalent to a hydrolysis of slightly more than 90 per cent of the ester. A similar experiment, in which the acidity developed was neutralized by calcium hydroxide, indicated a hydrolysis of approxi-

mately 94 per cent in 2 hours. In still other experiments, it has been possible to isolate adipic acid from the products of hydrolysis of hydrogen ethyl adipate by lipase, thus affording additional data in support of the conclusion that acid ethyl adipate, in contrast to acid ethyl malonate, is readily hydrolyzed by lipase. It should be noted here, that we have, in the present series, repeated our earlier work on the enzymatic hydrolysis of acid ethyl malonate and have again been unable to demonstrate any cleavage of the ester by the lipase preparation.

*Sodium Ethyl Adipate (Table II).*—Kastle (4) observed that none of the salts of acid esters was hydrolyzed by lipase (hepatic

TABLE II.

*Hydrolysis of 0.025 N Sodium Ethyl Adipate by Lipase.*

25 cc. portions of the ester were used. For complete saponification of this amount of ester 3.31 cc. of 0.1000 N NaOH were required.

Time.....5 min.	10 min.	20 min.	30 min.	1 hr.	2 hrs.	5 hrs.
Standard NaOH required for neutralization of acidity.						
cc.	cc.	cc.	cc.	cc.	cc.	cc.
0.02	0.02	0.02	0.02	0.10	0.10	0.21
	0.07	0.07	0.07	0.13	0.20	0.27
		0.15	0.15	0.15	0.27	0.34
			0.30	0.30	0.40	0.45
				1.00	1.09	1.09
					2.23	2.32
						2.87

and pancreatic). He considered that this was due to the fact that in aqueous solution the salts of the monoalkyl esters were ionized, while the dialkyl esters were not, and upon the assumption that the hydrolysis of a substance by means of an enzyme is always preceded by combination of the enzyme with the substrate, he interpreted his results as indicating that lipase cannot form combinations with ions, but only with molecules. The esters investigated by Kastle which are of interest in connection with the present work were sodium ethyl succinate and sodium ethyl fumarate. We have previously reported (1) that potassium ethyl malonate is not acted upon by lipase. Since, in contrast to hydrogen ethyl malonate, hydrogen ethyl adipate was readily

split by lipase, it was considered of particular interest to study the behavior of the metallic salts of the monoethyl ester of adipic acid towards lipase. If the failure of hydrolysis of potassium ethyl malonate was due, as suggested by Kastle, to ionization of the salt, and not, as we have believed, to the difficulty of removing the second ethyl group of diethyl malonate, then sodium ethyl adipate should not be hydrolyzed by lipase. If, on the other hand, the nature of the organic acid of the ester is the determining factor, sodium ethyl adipate should be hydrolyzed, since the enzymatic hydrolysis of diethyl adipate has been shown to proceed with the ready removal of both ethyl groups. As shown in Table II,

TABLE III.

*Hydrolysis of 0.025 N Diethyl Fumarate by Lipase.*

25 cc. portions of the ester were used. For complete saponification of this amount of ester 6.27 cc. of 0.1000 N NaOH were required.

Time.....30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	7 hrs.
Standard NaOH required for neutralization of acidity.						
cc.	cc.	cc.	cc.	cc.	cc.	cc.
1.39	2.56	3.06	3.14	3.14	3.21	3.21
	1.95	3.06	3.16	3.16	3.23	3.23
		2.60	3.14	3.14	3.22	3.22
			2.86	3.13	3.19	3.19
				2.97	3.16	3.22
					3.08	3.20
						3.09

sodium ethyl adipate was readily split in the presence of lipase. In two experiments, 67 (Table II) and 80 (not recorded in the tables) per cents of the ester were shown to undergo hydrolysis in 2 hours. This is in sharp contrast to the inactivity of lipase towards potassium ethyl malonate.

*Diethyl Fumarate (Table III) and Diethyl Maleate (Table IV).*—In view of the marked differences in the chemical properties of maleic and fumaric acids, the isomeric unsaturated dibasic acids related to succinic of the saturated series, a comparison of the hydrolysis of their diethyl esters by lipase was made. Diethyl fumarate was readily split, the progress of the reaction resembling that obtained with the related succinic acid; *i.e.*, equilibrium was

reached when one ethyl group was removed and further hydrolysis proceeded very slowly (Table III). The enzymatic hydrolysis of diethyl maleate took place slowly (Table IV) and even after 24

TABLE IV.

*Hydrolysis of 0.025 N Diethyl Maleate by Lipase.*

25 cc. portions of the ester were used. For complete saponification of this amount of ester 6.61 cc. of 0.1000 N NaOH were required.

Time....30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	7 hrs.	10 hrs.	24 hrs.
Standard NaOH required for neutralization of acidity.								
0.45	0.68	0.93	1.18	1.41	1.63	1.93	2.24	2.73
	0.50	0.78	1.05	1.29	1.51	1.82	2.15	2.63
		0.60	0.90	1.17	1.41	1.76	2.11	2.65
			0.65	0.97	1.21	1.56	1.94	2.52
				0.69	0.98	1.35	1.76	2.37
					0.73	1.14	1.58	2.23
						0.77	1.17	1.85
							0.87	1.58
								1.06

TABLE V.

*Hydrolysis of 0.025 N Diethyl Maleate plus 0.025 N Diethyl Adipate by Lipase.*

25 cc. portions of each ester were used. For complete saponification of the mixture of esters 12.94 cc. (6.27 and 6.67 cc.) of 0.1000 N NaOH were required.

Time.....30 min.	1 hr	2 hrs.	3 hrs	4 hrs.	5 hrs.	10 hrs
Standard NaOH required for neutralization of acidity.						
4.44	5.05	5.42	5.75	6.21	6.45	7.12
	5.60	6.02	6.35	6.76	7.09	7.66
		6.15	6.52	7.00	7.31	7.91
			6.29	6.81	7.11	7.75
				6.46	6.96	7.59
					6.49	7.32
						6.58

hours, only 16 per cent of the ester was split. It was considered possible that there might be present in the ester traces of some impurity, whose presence tended to inhibit the activity of the

lipase. However, when ethyl adipate was mixed with ethyl maleate, the enzymatic hydrolysis of the adipate proceeded normally (Table V). Thus after 3 hours, 5.23 cc. of alkali were re-

TABLE VI.

*Hydrolysis of 0.025 N Diethyl Maleate plus 0.025 N Diethyl Fumarate by Lipase.*

25 cc. portions of each ester were used. For complete saponification of the mixture of esters 12.82 cc. (6.55 and 6.27 cc.) of 0.1000 N NaOH were required.

Time....30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	7 hrs.
Standard NaOH required for neutralization of acidity.						
cc.	cc.	cc.	cc.	cc.	cc.	cc.
2.29	3.51	4.01	4.37	4.69	4.99	5.42
	2.96	3.73	4.13	4.44	4.73	5.18
		3.43	3.94	4.27	4.81	5.08
			3.58	3.98	4.39	4.82
				3.58	4.03	4.43
					3.66	4.15
						3.68

TABLE VII.

*Hydrolysis of 0.025 N Diethyl dl-Maleate by Lipase.*

25 cc. portions of the ester were used. For complete saponification of this amount of ester 5.76 cc. of 0.1000 N NaOH were required.

Time....30 min	1 hr.	2 hrs.	3 hrs.	5 hrs.	7 hrs.	10 hrs.	24 hrs.
Standard NaOH required for neutralization of acidity.							
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
0.07	0.37	0.77	1.22	1.82	2.25	2.35	2.45
	0.15	0.59	1.01	1.64	2.21	2.38	2.49
		0.30	0.74	1.33	2.04	2.33	2.47
			0.45	1.07	1.82	2.32	2.47
				0.60	1.31	2.09	2.40
					0.87	1.67	2.34
						1.20	2.34
							1.95

quired to neutralize the acidity developed when the lipase reacted with ethyl adipate alone, 0.65 cc. with ethyl maleate alone, and 6.29 cc. when the enzyme acted upon a mixture of the two esters.

Similarly the presence of diethyl maleate did not retard the hydrolysis of the isomeric fumarate (Table VI). After 3 hours, the acidities developed were equivalent to 0.65, 2.86, and 3.58 cc. of standard alkali respectively, in the case of the maleate alone, fumarate alone, and a mixture of equal parts of maleate and fumarate. The sum of the acidities developed by action of the lipase on the esters separately, 3.51 cc., compares well with the figure obtained from the mixture of esters, 3.58 cc. It is evident that the slow rate of hydrolysis of diethyl maleate by lipase cannot be explained by destruction or retardation of the activity of the enzyme by the ester itself, impurities present, or the maleic acid formed.

*Diethyl dl-Malate (Table VII).*—The hydrolysis of the diethyl ester of *dl*-malic acid, the monohydroxy derivative of succinic acid, by lipase was also very slow. Similar results were obtained in unpublished experiments by Christman and one of us (L.) with diethyl tartrate, the ester of the dihydroxy derivative of succinic acid.

#### DISCUSSION.

It is of interest to compare the results obtained in the present series of papers on the enzymatic hydrolysis of the esters of the dicarboxylic acids with those obtained by others who have studied the velocity of hydrolysis of the esters of these acids by acids and alkalies. The investigations of Knoblauch (5) on the velocity of saponification by alkali of the esters of the polybasic acids have stimulated much further study, particularly by Skrabal and his associates. Skrabal (6) observed that the velocity constant for alkaline saponification of the first alkyl group of dimethyl oxalate was about 10,000 times greater than that for the second alkyl group. In a later study (7), he compared the velocity coefficients for the alkaline saponification of a series of dialkyl esters of the dicarboxylic acids. The ratio between the saponification coefficients of the first and second alkyl groups was greatest in the case of oxalic acid, and as the number of carbon atoms which separated the two carboxyl groups increased, the ratio tended to decrease and, for the higher members of the series, to approach 2. Thus for the esters of oxalic, malonic, succinic, and glutaric acids, the ratios between the two saponification constants were 17,000,

80, 7, and 6 respectively. This greater difficulty in the removal of the second alkyl group by alkaline hydrolysis parallels our results on the enzymatic hydrolysis of the esters of malonic and succinic acids. There was, however, no marked difference in the velocity coefficients of alkaline hydrolysis of the esters of succinic acid and the next higher member of the series, glutaric acid, which is parallel to the difference in the hydrolysis of these two esters which we have observed under conditions of enzymatic hydrolysis.

The difference in the acidities of the acid esters of malonic and adipic acids might also explain the difference in their behavior in enzymatic hydrolysis. It was considered possible that the acidity developed in the hydrolysis of diethyl malonate to acid ethyl malonate might be sufficient to destroy or inhibit the enzyme. However, in an experiment in which the reaction mixture was buffered by a phosphate buffer (Sørensen) to maintain a constant pH of 7.0,<sup>1</sup> the course of the hydrolysis of diethyl malonate by lipase was not altered; *i.e.*, the reaction reached an equilibrium when the acidity developed was equivalent to the removal of one ethyl group. Moreover, according to Walker (8), the dissociation constants of ethyl hydrogen succinate and ethyl hydrogen adipate do not differ sufficiently to explain the results with diethyl succinate and diethyl adipate, on the basis of the greater acidity of the acid ester of the former.

The velocity coefficients of saponification for the diethyl esters of fumaric acid and the saturated dicarboxylic acids paralleled the dissociation constants of the acids themselves (7, 9). As predicted from a comparison of the dissociation constants of the first carboxyl groups of maleic and fumaric acids, the velocity coefficient for the alkaline saponification of the first ethyl group of diethyl maleate should have been greater than the corresponding coefficient of diethyl fumarate. In fact, however, the alkaline hydrolysis of diethyl maleate proceeded much more slowly than that of diethyl fumarate (9). No explanation for this anomalous behavior of the ester of maleic acid was evident. We have found similarly that the enzymatic hydrolysis of diethyl maleate proceeds much more slowly than that of diethyl fumarate. Since maleic acid

<sup>1</sup> This experiment was carried out by Dr. J. L. McGhee of Emory University, in the laboratory of the senior author (L.) during the summer of 1925.



and its acid ethyl ester are much more strongly acidic than fumaric acid and its acid ester (8), the possibility was suggested that the results observed might be explained by the different acidities developed in the two enzymatic reactions. However, when the solution of maleic ester was buffered to maintain a pH of 7.0, the velocity of the hydrolysis of diethyl maleate by lipase was not increased.

Calcium chloride has been reported (10) to accelerate the activity of lipolytic enzymes due to the formation of insoluble or slightly ionized calcium salts of the fatty acids produced by the hydrolysis of the ester. In our experiments, however, the addition of calcium chloride in equimolar amounts to diethyl adipate did not influence the velocity of the hydrolysis by lipase.

In connection with the enzymatic hydrolysis of acid ethyl adipate and sodium ethyl adipate (Tables I and II), it should be noted that, although hydrolysis proceeded readily after the addition of the enzyme, if the acidity developed was neutralized at any point, further hydrolysis ceased almost completely. This is in harmony with our earlier observations with diethyl glutarate and adipate (2). If the acidity developed in the hydrolysis of these *diethyl* esters was neutralized before 50 per cent hydrolysis (*i.e.* corresponding to removal of one ethyl group) had been obtained, the reaction continued, but if the neutralization of the acidity occurred after a hydrolysis of more than 50 per cent of the ester no further hydrolysis occurred. The interpretation of these phenomena is reserved for further investigation in this laboratory.

#### SUMMARY.

1. In contrast to acid ethyl malonate and potassium ethyl malonate, acid ethyl adipate and sodium ethyl adipate have been shown to be readily hydrolyzed by the lipase of hog liver. The failure of hydrolysis of potassium ethyl malonate is, therefore, not considered to be due to the ionization of the salt of the ester and the inability of lipase to combine with ions, as suggested by Kastle (4), but rather to the fact, as shown previously (1), that the second ethyl group of diethyl malonate is resistant to hydrolysis by lipase. Both ethyl groups of diethyl adipate are readily removed by hydrolysis by lipase and the acid ester and its salts are also readily hydrolyzed by the enzyme.

2. The course of the hydrolysis of diethyl fumarate by liver lipase was similar to that observed in the hydrolysis of the ester of the related saturated acid, succinic; *i.e.*, equilibrium was reached when a hydrolysis corresponding to the removal of one ethyl group had occurred. The isomeric diethyl maleate was, however, only slowly split by lipase. The presence of diethyl maleate did not influence unfavorably the hydrolysis of its isomer, diethyl fumarate.

3. The enzymatic hydrolysis of the diethyl ester of *dl*-malic acid, monohydroxysuccinic, proceeded much less rapidly than the hydrolysis of the diethyl ester of succinic acid.

4. The relation of the reactions in the enzymatic hydrolysis of the dialkyl esters of the dicarboxylic fatty acids to those occurring under conditions of alkaline hydrolysis is discussed and certain differences are pointed out.

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# A STUDY OF THE MOLYBDIC OXIDE COLORIMETRIC METHOD FOR THE ESTIMATION OF THE PHOSPHORUS COMPOUNDS OF THE BLOOD.

BY JOSEPH H. ROE, OLIVER J. IRISH, AND JAMES I. BOYD.

*(From the Department of Chemistry, George Washington University Medical School, Washington.)*

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The molybdic oxide colorimetric method for the estimation of phosphorus was introduced by Taylor and Miller (1) in 1914. These authors precipitated the phosphate from an ashed sample as ammonium phosphomolybdate, and treated for color production by reducing the phosphomolybdate with phenylhydrazine. Bell and Doisy (2), in 1920, showed that a selective reduction of molybdenum present as ammonium phosphomolybdate could be carried out in an excess of molybdate ions, and developed a method which avoids the necessity of isolating the phosphorus as ammonium phosphomolybdate. Briggs (3), in 1922, introduced the modification of producing the color in an acid medium, claiming greater stability in the color obtained. In 1924, Benedict and Theis (4) modified this procedure still further by using molybdic acid instead of the ammonium salt and boiling the molybdate-phosphate mixture to complete the reaction.

Recently Stanford and Wheatley (5) have shown that the degree of acidity markedly influences the color intensity in the Briggs method. These authors state that the depth of color increases with gradual increase in acidity to a maximum, and then decreases; and that, in the region of the maximum, the color is less stable than when in the paler region. They conclude that, "It is necessary to compromise between the degree of acidity giving the maximum coloration and the degree of acidity which gives the maximum stability, because at maximum stability the coloration is too pale for satisfactory colorimetric comparison," and they recommend working in the region of 0.5 N sulfuric acid.

In studying the molybdic oxide color production we found the

following conditions must be regulated: (1) the concentration of molybdic acid used; (2) the concentration of hydroquinone; (3) the time allowed for completing the reaction; (4) the hydrogen ion concentration; (5) the presence of salts; (6) the amount of phosphorus in the sample being analyzed.

Color production in the molybdic oxide method is dependent upon a difference in the velocities of the two reactions: (1) the reduction of molybdic acid, and (2) the reduction of phosphomolybdic acid. The reduction of phosphomolybdic acid in the presence of molybdic acid is "selective," as claimed by Bell and Doisy (2), only in the sense that phosphomolybdic acid is more easily reduced than molybdic acid. When excess molybdic acid is present, it is reduced as well as the phosphomolybdic acid, and deeper blue colors are obtained, which do not have a quantitative relationship to the amount of phosphorus present. Similarly, if a strong reducing agent, such as stannous chloride, or excess of a mild reducing agent like hydroquinone, is present, molybdic acid as well as phosphomolybdic acid, is reduced, and the color produced is again not proportional to the absolute amount of phosphorus. It is therefore important to use amounts of molybdic acid safely below the concentrations that will give color in controls where no phosphorus is present. It is necessary also to limit the concentration of the reducing reagent used to an amount that will not give a coloration with molybdic acid free from phosphorus, and yet will be sufficient to reduce quantitatively the phosphomolybdic acid. Conditions are best adjusted to obtain results proportional to the absolute amount of phosphorus present by using as a criterion the development of a color with 0.025 mg. of phosphorus that will read the same in the colorimeter as that produced by twice as much phosphorus (0.05 mg.) treated similarly, but diluted to twice the volume before comparison.

Since the reaction is comparatively slow, we prefer the Benedict and Theis modification of boiling the mixtures upon a water bath to develop the color. This gives a more intense and more stable color than is obtained by the Briggs (3) or Bell and Doisy (2) methods of color production in the cold. The color obtained by boiling is highly stable. It grows deeper very slowly upon standing, but will maintain its optical qualities for days. We have found that 10 to 15 minutes boiling upon a water bath produces colors proportional to the amount of phosphorus present, but prolonged

boiling destroys quantitative relationship to the absolute amount of phosphorus. This is explained by the fact that continued boiling will bring about a reduction of molybdic acid, as is shown by control experiments, with no phosphorus present.

The influence of acidity is shown by the curve (Fig. 1) where colorimeter readings are plotted against normality. Maximum coloration occurs around 0.3 normality, in which region the curve is very sharp. In the zone between 0.9 N and 1.9 N there is no change in the intensity of the colors produced. This zone is a very satisfactory region for color production in standard and unknown solutions in which it is obviously difficult always to produce the same acidity. We, therefore, introduce into our standard enough sulfuric acid to bring it to the mid-point of this range (1.4 N), and when ashing use enough sulfuric acid to approach this normality after the average amount required for oxidation is spent. The method of Benedict and Theis (4) for inorganic phosphorus requires color production in the non-variable range of intensities with reference to acidity, but practically all of the methods for total phosphorus or inorganic phosphorus carry out color production in acidities which may yield considerable error.

It has been shown by Denis (6) that the salts used as anticoagulants will interfere when present in excess; and by Rimington (7) that ammonium sulfate in concentrations greater than 1.4 normality markedly decreases the velocity of color production. We have experienced similar interference from sodium sulfate present in quantities greater than 0.02 N, and from mere traces of nitrates, but have found no interference from ammonium sulfate or sodium sulfate in the concentrations found in the blood or from the amount of potassium oxalate regularly used as an anticoagulant.

In our experience the most favorable results will be obtained by working with samples containing 0.03 mg. to 0.05 mg. of phosphorus. Below this range there is a plus error, and above it a minus variation occurs.

We have tried many ashing procedures, both wet and dry, and have found the use of a mixture of sulfuric and nitric acid, with the introduction of a few drops of redistilled superoxol late in the reaction to be the most satisfactory method. The use of superoxol was introduced by Baumann (8), who uses sulfuric acid and superoxol only. When ashing whole blood a small amount of nitric acid facilitates the oxidation very desirably, but nitric acid is

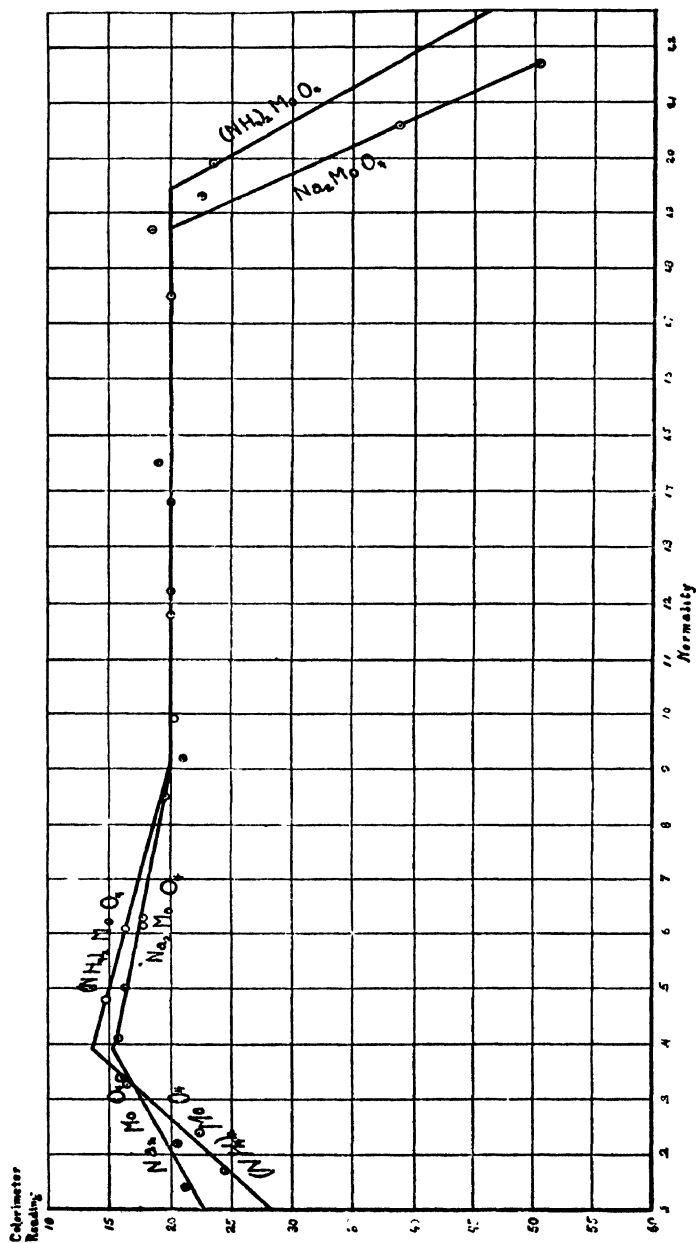


FIG. 1. Relation of acidity to molybdenum oxide color production.

unsatisfactory for completing the oxidation. Nitric acid tends to boil out early in the reaction, even though large excesses are taken, and, if added later in the procedure to the dry sulfuric acid mixture, a yellowish nitric acid compound, possibly a nitrated phenol, is formed, which interferes with subsequent color production, giving pale blue or green colors. Bloor (9) and other workers have called attention to the difficulty of removing nitric acid in this ashing procedure. Nitric acid can be removed completely by boiling as long as the mixture contains water, but from a dry sulfuric acid mixture nitric acid cannot be removed satisfactorily, and should never be present in the last stages of the ashing procedure.

We have found in agreement with Baumann (8) that losses of phosphoric acid from volatilization often occur when the acid mixture is heated with a flame applied directly. Temperature control is therefore absolutely necessary. The most satisfactory way to accomplish this is to place the acid mixture in a bath in which the temperature can be regulated and never allowed to go above 200°. If the temperature is permitted to go much higher than this, losses of phosphoric acid from volatilization will occur, and there will also be conversion to meta- and pyrophosphoric acid.

We disagree with Baumann (10) where he claims that losses of phosphoric acid from volatilization when boiled with sulfuric acid will occur below 200°C. We have repeatedly run controls in which known amounts of phosphate were boiled at 180–200°C. with the amounts of sulfuric and nitric acid regularly used in ashing whole blood, and have not experienced losses greater than the experimental errors that normally occur in colorimetric measurements. Table I shows a typical set of recoveries from such controls.

TABLE I.

*Recoveries of Phosphorus after 3 Hours Boiling with Sulfuric and Nitric Acid at 180–200°.*

Phosphorus taken.	Phosphorus recovered.	Error.
<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
0.025	0.0250	0
0.025	0.0240	–4.0
0.025	0.0243	–2.4
0.025	0.0250	0
0.025	0.0268	+7
0.025	0.0243	–2.4
0.025	0.0255	+2



*Technique for Total Phosphorus in Whole Blood.*

Into a 25 cc. graduated cylinder, or volumetric flask, partly filled with phosphate-free water, pipette 2 cc. of whole blood. Make up to the mark with water and mix thoroughly. Place 2 cc. of this mixture in a 20 × 200 mm. graduated test-tube, and add 1 cc. of a mixture of seven parts of sulfuric acid and three parts of nitric acid. Place in a temperature-controlled bath, which is set at around 180°, and heat until all water has been driven off and a brownish charred mixture remains. This will require 20 to 30 minutes. At this stage add slowly from a pipette redistilled superoxol, drop at a time, until the mixture apparently clears, and continue heating until all water vapor is driven off. The mixture will char again slightly. Add superoxol a second time, drop at a time, until the mixture becomes perfectly clear. Continue heating at 180° for about 15 minutes to remove all traces of superoxol, then remove the tube, cool, and add 10 cc. of water. The entire oxidation procedure should consume about 1 hour.

Into a second graduated test-tube place 10 cc. of standard phosphate solution containing 0.05 mg. of phosphorus, and add 0.5 cc. of concentrated sulfuric acid. Then to the standard and unknown tubes add 1 cc. of 5 per cent ammonium molybdate and 1 cc. of 0.5 per cent hydroquinone in 15 per cent sodium bisulfite reagent. Place the tubes in a boiling water bath for 10 minutes; remove and cool. Dilute the standard to 25 cc. and the unknown to a volume that will give a color approximately matching the standard (25 cc. for normal bloods), and compare in a colorimeter in the usual manner.

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## A COLORIMETRIC METHOD FOR THE ESTIMATION OF BLOOD CALCIUM.

By JOSEPH H. ROE AND BERNARD S. KAHN.

(From the Department of Chemistry, George Washington University Medical School, Washington.)

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### *Principle.*

The calcium is precipitated as calcium phosphate from an alkalized trichloroacetic acid serum filtrate and estimated as phosphate by the Benedict and Theis (1) molybdic oxide colorimetric method, slightly modified.

### *Procedure.*

Place 2 cc. of blood serum in a small flask and add 4 cc. of distilled water and 4 cc. of 20 per cent trichloroacetic acid. Mix thoroughly, allow to stand 10 minutes, and filter through a double acid-washed calcium-free filter paper. Transfer 5 cc. of the trichloroacetic acid filtrate to a 15 cc. conical centrifuge tube which has been thoroughly cleaned by immersion in bichromate sulfuric acid "cleaning solution" for several hours. Place 1 drop of 1 per cent phenolphthalein in the tube and add, drop at a time, 20 per cent calcium-free sodium hydroxide until a definite pink color is obtained. Add 1 cc. of 1 per cent trisodium phosphate, swirl the tube until thoroughly mixed, cork, and set aside for 1 hour.

After 1 hour's standing, centrifuge for 3 minutes. Decant carefully the supernatant fluid from the calcium phosphate precipitate. Place the inverted tube upon a pad of filter paper to drain for 2 or 3 minutes, then wipe away adherent solution from the mouth of the tube with a clean cloth or paper. Wash twice with 5 cc. portions of 50 per cent alcohol made faintly alkaline to phenolphthalein with a few drops of calcium-free alkali. In washing, the mat of calcium phosphate in the bottom of the tube must be

thoroughly broken up with a glass stirring rod, and the process of centrifuging, decanting, and draining the tube should be carried out as described above. Dissolve the washed precipitate in 5 cc. of 5 per cent sulfuric acid by volume (5 cc. concentrated  $\text{H}_2\text{SO}_4$  per 100 cc. of water), and decant into a Rothberg-Evans sugar tube, or a graduated test-tube; wash the centrifuge tube twice with approximately 3 cc. and 2 cc. portions of the 5 per cent sulfuric acid, adding the washings to the graduated tube.

In a similarly graduated tube place 10 cc. of standard phosphate solution containing 0.05 mg. of phosphorus, and add 0.5 cc. of concentrated sulfuric acid. Now add to each tube 1 cc. of 5 per cent sodium molybdate and 1 cc. of hydroquinone bisulfite reagent. Place the tubes in a boiling water bath for 10 minutes. Remove, cool, dilute the standard to 15 cc. and the unknown to a volume giving a color that will approximately match the standard (15 cc. in normal bloods), and compare in a colorimeter in the usual manner.

#### Calculation.

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.05 \times \frac{\text{dilution of unknown}}{\text{dilution of standard}} \times \frac{60}{31} \times 100 =$$

mg. Ca per 100 cc. of serum.

#### Reagents.

1. *Standard Phosphate Solution.*—For convenience we use the Bell-Doisy phosphate standard solutions, since these are practically always in use in the laboratory for determining inorganic phosphorus.

(a) *Stock Solution.*—Dissolve 4.394 gm. of pure dry monopotassium phosphate in 1 liter of phosphate-free water. 1 cc. of this reagent contains 1 mg. of phosphorus. Preserve with chloroform.

(b) *Phosphate Solution for Calcium Estimation.*—Pipette accurately 5 cc. of the stock phosphate solution into a liter flask, and make up to the mark with phosphate-free water. 10 cc. of this solution contain 0.05 mg. of phosphorus, equivalent to 0.097 mg. of calcium as  $\text{Ca}_3(\text{PO}_4)_2$ .

2. *Molybdate Solution.*—A 5 per cent solution of pure sodium molybdate ( $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ) is used. Pure ammonium molybdate of the same concentration may be used.

3. *Hydroquinone Bisulfite Reagent.*—Dissolve 30 gm. of pure sodium bisulfite and 1 gm. of highest purity hydroquinone in 200 cc. of phosphate-free water.

## EXPERIMENTAL AND DISCUSSION.

The authors at first checked the above procedure against the Kramer-Tisdall (2) and the Fiske (3) methods for estimating blood calcium, and obtained results that agreed satisfactorily. Since these two methods are titration methods which, necessarily, involve personal error in judging end-points, and with which it is difficult to obtain close checks, we discontinued testing against these methods and tried the colorimetric procedure upon known calcium solutions.

TABLE I.

Serum No.	Ca per 100 cc.	Serum plus 4 mg. calcium.			Serum plus 10 mg. calcium.		
		Calculated.	Recovered.	Recovered.	Calculated.	Recovered.	Recovered.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>	<i>mg</i>	<i>mg.</i>	<i>per cent</i>
1	11.8	15.8	16.1	102	21.8	21.5	98.6
2	10.3	14.3	14.2	99.3			
3	9.2	13.2	13.0	98.4			
4	10.1				20.1	19.7	98.0
5	10.7				20.7	19.8	95.6

TABLE II.

Serum No.	Ca per 100 cc. serum.	Ca per 100 cc. serum diluted 2.5 (calculated).	Ca per 100 cc. serum diluted 1:1 (calculated).	Ca per 100 cc obtained by analysis	Recovered.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
1	10.1	4.04		4.1	101.4
2	10.7	4.28		4.2	98.1
3	10.3	4.12		4.1	99.5
4	11.5		5.75	5.79	100.6
5	9.2		4.60	4.80	104.3
6	9.9		4.95	4.98	100.6
7	11.8		5.90	5.70	96.6

With a calcium solution containing 10 mg. per 100 cc. we obtained 98 to 100 per cent recoveries. The results were equally as satisfactory when varying amounts of calcium were added to blood serum. We also obtained quantitative recoveries with sera diluted to contain one-half and two-fifths the normal blood calcium, thus demonstrating the applicability of the method to hypocalcemic conditions of 4 to 6 mg. per 100 cc. of serum. Typical protocols are shown in Tables I and II.

Since the method is dependent upon the fact that calcium phosphate has a low solubility in an alkaline medium, experiments were carried out to determine the conditions that will give the least solubility. The influence of concentrating the alkali was first investigated, and it was found that the lowest solubility exists in hydrogen ion concentrations ranging from pH 7 to pH 12. In this range  $\text{Ca}_3(\text{PO}_4)_2$  is soluble to the extent of 1.2 to 1.8 mg. per 100 cc. of solution, as shown by the curve in Fig. 1. Above pH 12 the curve rises sharply, showing an increase in solubility to 12.9 mg. per 100 cc. of solution at pH 14. Hence the trichloroacetic

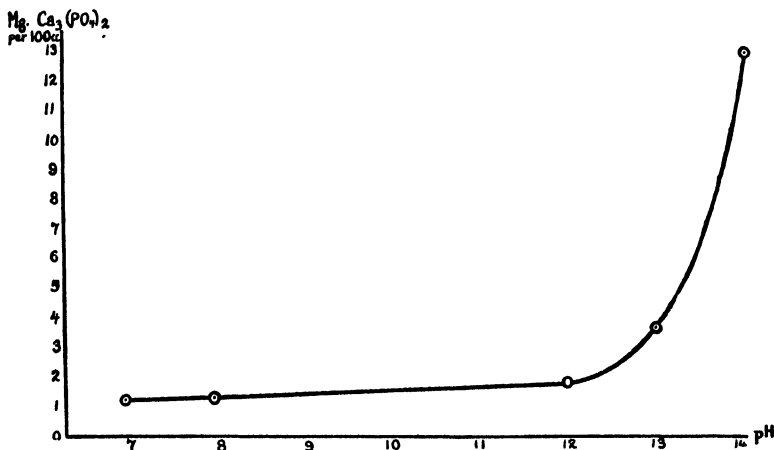


FIG. 1. Curve showing solubility of  $\text{Ca}_3(\text{PO}_4)_2$  at varying hydrogen ion concentrations.

acid filtrate is alkalinized until it reaches a hydrogen ion concentration just basic to phenolphthalein in order to obtain maximum precipitation.

We examined various substances that could be used for washing the  $\text{Ca}_3(\text{PO}_4)_2$  free from the excess soluble phosphate used as a precipitant. The lowest solubility was found with mixtures of alcohol and water. In 50 and 85 per cent alcohol the solubilities determined by the colorimetric method were 0.7 mg. and 0.5 mg. of  $\text{Ca}_3(\text{PO}_4)_2$  per 100 cc. of solution. These mixtures also proved to be satisfactory washes and were therefore adopted for this purpose.

Analyses carried out upon phosphate precipitates from known calcium solutions to determine the comparative losses from solubility in alcohol and water gave the following results: When washed with 50 per cent alcohol, precipitates representing 10 mg. of calcium per 100 cc. never gave less than 9.8 mg. after two washings, and 9.7 mg. after four washings. Similar experiments, using distilled water, gave 9.0 mg. after two washings, and 8.3 mg. after four washings. We do not feel that the small losses experienced when using 50 per cent alcohol for washing justify the introduction of a correction factor.

The magnesium of the blood does not interfere for either of two reasons: (1) The trichloroacetic acid filtrate is first alkalinized, and, as the solubility of magnesium hydroxide is 0.9 mg. per 100 cc., this step would tend to remove magnesium as the hydroxide, if present to that extent; (2) magnesium phosphate has a solubility of 20.5 mg. per 100 cc., and as this amount is very much in excess of the amount of magnesium found in the blood, there is no phosphate precipitated as magnesium phosphate. We demonstrated that no interference occurs by an experiment in which analyses were carried out upon four samples of pure calcium solution and upon four similar quantities of the same solution to each of which was added 2 mg. of magnesium per 100 cc. The values obtained for calcium were the same in the magnesium additions as in the controls.

Duplicate determinations by this method agree excellently. The technique of precipitating and washing the  $\text{Ca}_3(\text{PO}_4)_2$  gives practically constant results. Inspection of the calculation formula shows that a variation of 1 mg. in the result will require a difference in colorimeter readings of 2 mm. This is a very liberal margin for errors in technique as compared with conditions that will change results obtained by the titration methods.

Since the proposed method is colorimetric, it conforms to the requirements of a micro method. The equivalent of 1 cc. of serum is used in the regular procedure. This amount can be reduced to the equivalent of  $\frac{1}{2}$  or  $\frac{2}{3}$  of a cc. without loss of accuracy, as was shown in the experiments with diluted serum. In the Kramer-Tisdall (2) method, 2 cc. of serum is the amount regularly used for the analysis. This amount will give a burette reading around 1 cc. of 0.01 N  $\text{KMnO}_4$  with normal bloods. If 1 cc. of

normal serum, or 2 cc. of a hypocalcemic serum containing 5 mg. of calcium per 100 cc. are being analyzed, the titration with 0.01 N  $\text{KMnO}_4$  will be 0.5 cc. If  $\frac{2}{3}$  of a cc. is analyzed, the burette reading will be 0.2 cc. It is obvious that accurate results cannot be obtained when such small quantities of the titrating reagent are used, and that the colorimetric method is capable of being adapted to the analysis of much smaller amounts of serum than the titration methods.

This procedure requires no more skill on the part of the operator than the methods in use at present, and is equally as rapid. It does not necessitate frequent checking of reagents like the permanganate or the alkalimetric titration methods. It is applied to the same trichloroacetic acid filtrate that is used in the determination of inorganic phosphorus, and, as blood calcium and phosphorus are usually studied together, this is a convenient adaptation. It is, therefore, a desirable method for clinical investigation.

The important requirements of this method are pure reagents and the ability to determine inorganic phosphorus accurately. All reagents and apparatus must be calcium-free previous to the precipitation of the calcium phosphate, and, after this, they must be phosphate-free. Most grades of filter paper, even acid-washed, contain calcium, and will require considerable washing before they can be used in preparing the trichloroacetic acid serum filtrate. It is obviously very important to separate the precipitated proteins either by centrifuging, or by using a filter paper of a grade whose washings give negative tests for calcium (Whatman's No. 42). Hydroquinone and sodium molybdate of the highest purity should be used. Many c.p. sodium hydroxide preparations on the market contain considerable calcium. No alkali should be used unless it does not give a precipitate of calcium phosphate when brought to a hydrogen ion concentration slightly alkaline to phenolphthalein (approximately pH 8.5) and soluble phosphate is added in excess. It is important to note that the solubility of calcium phosphate increases with alkalinity, a sharp rise in the solubility curve occurring around pH 13 to 14. A negative result when soluble phosphate is added to a strong solution of alkali therefore does not necessarily indicate that the solution is free from calcium.

## SUMMARY.

1. A colorimetric method for estimating blood calcium has been developed which is based upon the precipitation of calcium as phosphate, and the determination of the latter by the molybdic oxide colorimetric procedure.

2. The method is very accurate and is a successful micro procedure, being adaptable to much smaller quantities of serum than other methods in use at the present time.

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## CONCENTRATED ANTINEURITIC VITAMIN PREPARED FROM BREWERS' YEAST.\*

By ATHERTON SEIDELL.

(From the Hygienic Laboratory, United States Public Health Service, Washington.)

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Although the method previously described<sup>1</sup> for preparing concentrated antineuritic vitamin from brewers' yeast gave a highly active product it became desirable to obtain much larger quantities of it. A moderately large scale and somewhat less complicated process have, therefore, been developed. Furthermore, the active material has been obtained in the form of a stable, dry, non-hygroscopic powder.

*Preparation of the Vitamin Solution.*—Fresh ice-cold bottom yeast obtained from the brewery as a thick mush is mixed with about an equal volume of water and heated, with constant stirring, to about 90°C. essentially as done by Osborne and Wakeman.<sup>2</sup> After several minutes it is cooled to a moderate temperature and the coagulated protein and insoluble material removed by means of a Sharples supercentrifuge. Air-dried brewers' yeast may be used instead of the fresh material, in which case 1 kilo of the finely ground product is added to each 10 liters of boiling water and the insoluble part removed by means of the Sharples supercentrifuge.

*Preparation of "Activated Solid."*—To the clear, dark brown solution, separated from the coagulated protein as above mentioned, 30 gm. per liter of English fullers' earth<sup>3</sup> are added and

\* Approved for publication by the Surgeon General.

<sup>1</sup> Seidell, A., *J. Am. Chem. Soc.*, 1922, xlv, 2042.

<sup>2</sup> Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1919, xl, 383.

<sup>3</sup> The variety of fullers' earth believed to be best suited for adsorbing the antineuritic vitamin is that obtained from Surrey, England. It should be tested for its adsorptive capacity by adding to a suspension of 1.0 gm. of the sample in 50 cc. of water, 0.16 gm. of quinine bisulfate and agitating actively for  $\frac{1}{2}$  hour. The filtrate from this mixture should show no clouding upon the addition of five drops of Mayer's alkaloid reagent.

the mixture constantly stirred for at least  $\frac{1}{2}$  hour. The suspended fullers' earth, which adsorbs about 90 per cent of the antineuritic vitamin of the yeast solution, is removed by means of the Sharples supercentrifuge. It should be thoroughly dried in a current of warm air and finally ground and well mixed. The "activated solid" thus prepared contains usually about 2 per cent of nitrogen, which probably corresponds to a content of 12 to 15 per cent of organic matter. It protects pigeons from loss in weight, or restores that lost on an exclusive diet of polished rice, in doses of 0.100 to 0.200 gm. given on alternate days.

*Extraction of the Vitamin from Activated Solid.*—In the writer's previous work a saturated barium hydroxide solution was used for the purpose of liberating the vitamin from its combination with fullers' earth. It has been pointed out by Levene and van der Hoeven,<sup>4</sup> however, that barium hydroxide precipitates a portion, at least, of vitamin B from its aqueous solution. For the large scale extraction it was, therefore, decided to use sodium hydroxide instead of barium hydroxide and acidify the resulting extract with acetic acid instead of sulfuric.

As to the optimum concentration and total quantity of sodium hydroxide necessary to liberate as much vitamin as possible but not cause its destruction, accurate information has not as yet been obtained. The experiments so far made indicate that a good yield of a highly active product is obtained when 0.3 normal sodium hydroxide is used in the proportion of 1 liter per 100 gm. of activated solid.

Since the bowl of the Sharples supercentrifuge will contain only about 3.0 kilos of fullers' earth, the amount of activated solid to be used for each extraction should not exceed 2.5 kilos. This quantity was mixed with 25 liters of distilled water and an amount of very concentrated sodium hydroxide solution containing 300 gm. of NaOH was added. The mixture was actively agitated for 3 minutes and immediately run through the Sharples supercentrifuge in the shortest possible time. About 5 liters of distilled water were used to displace the vitamin solution from the apparatus. The clear effluent was received in a vessel to which had been added an amount of glacial acetic acid slightly in excess of the equivalent of sodium hydroxide used.

<sup>4</sup> Levene, P. A., and van der Hoeven, B. J. C., *J. Biol. Chem.*, 1925, lxx, 483.

*Concentration of the Vitamin Extract.*—One or more of the aqueous extracts thus prepared, each amounting to about 30 liters and obtained from 2.5 kilos of activated solid, are promptly concentrated by vacuum distillation to one-tenth or one-twentieth their volume. Using an efficient enamel-lined vacuum still<sup>5</sup> this can be accomplished within a few hours and at a temperature not exceeding 75°C. A considerable amount of a brownish precipitate separates during the concentration of the extract. This cannot readily be removed by filtration but may be satisfactorily thrown down by means of an ordinary cup centrifuge.

The clear solution decanted from the brown precipitate was brought to a measured volume and an aliquot used for determination of the sodium acetate present. This was done by evaporating, igniting, and titrating the ash with standard sulfuric acid, using methyl orange as indicator. On the basis of this result the calculated amount of concentrated sulfuric acid, required to convert the sodium into sulfate, was slowly added to the actively stirred solution. On standing in a cool place the larger part of the sodium sulfate crystallized as  $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ .

It has been found that of the total amount of sodium hydroxide used for the extraction of the vitamin, a considerable portion of it remains attached to the fuller's earth and does not appear in the aqueous extract. This amount increases with the amount of sodium hydroxide used. The process of liberation of the vitamin and other bases from the activated solid appears, therefore, to be in the nature of a replacement of the weaker organic bases by the stronger inorganic base.

The solution drained from the crystallized  $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$  was concentrated by vacuum distillation and again cooled for the crystallization of a further amount of the inorganic sulfate. The addition of an amount of methyl alcohol, to yield a concentration of about 20 to 30 per cent, caused the precipitation of what appeared to be a further amount of the same material which separates during the original evaporation of the aqueous extract of the activated solid. This may be best removed by a centrifuge. A further addition of methyl alcohol should not be made at this

<sup>5</sup> I am indebted to Dr. C. A. Browne, Chief of the Bureau of Chemistry, for permission to use the excellent equipment of the Color Laboratory at Arlington Farm.

stage since the active fraction begins to precipitate as the concentration of alcohol increases. The exact point between the end of the precipitation of the inactive material and the beginning of that of the active cannot be selected with certainty.

After removal of practically all of the sodium sulfate and as much as possible of the inactive fraction, the solution was evaporated under diminished pressure to a very thick viscous paste.

*Conversion of the Active Viscous Residue to a Non-Hygroscopic Condition by Means of Strong Alcohol.*—It has been pointed out by Levene and van der Hoeven<sup>1</sup> that the Osborne and Wakeman fraction, when shaken with absolute alcohol, acquires the character of a fine non-hygroscopic powder. This has been found to be the case with the viscous hygroscopic residue obtained as above described.

In the present case successive portions of strong alcohol (98 to 100 per cent) were added and after a short period of contact with the soft residue were decanted. The suspended flocculent material in the decanted alcohol soon subsides and the supernatant liquid may be easily poured off. This process was continued until no more of the viscous residue remained. The flocculent precipitate was washed by decantation with further amounts of strong alcohol and finally brought upon a Buchner funnel and sucked dry. It was then completely freed of alcohol by vacuum desiccation and was found to possess none of the hygroscopic character of the viscous concentrate from which it was prepared.

The alcoholic solutions, decanted from the flocculent precipitate, when evaporated by vacuum distillation and the resulting viscous material again treated with very strong alcohol, yield a further amount of the non-hygroscopic powder. The last portions of viscous solids are, however, not easily converted to the non-hygroscopic condition.

Methyl alcohol may be used in place of ethyl for converting the material to a powder, especially in the beginning. The product is, however, undoubtedly more soluble in methyl than ethyl alcohol and additional quantities of it can be obtained by means of ethyl alcohol after as much as possible has been converted to the flocculent condition with the aid of methyl alcohol.

As mentioned above the active fraction begins to precipitate before all of the inactive portion has been removed from the solu-

tion. Consequently, an amount of the active material will be lost varying with the stage at which its collection is begun. It is, therefore, not possible to obtain a constant yield of the active fraction without further standardization of the details of the process.

Controlling the separations by means of pigeon feeding tests<sup>6</sup> it has been found that from 5 kilos of activated solid (correspond-

TABLE I.

*Change in Weight of Pigeons Fed on Polished Rice and Given on Alternate Days the Specified Doses of Several Preparations Obtained in Connection with the Concentration of the Antineuritic Vitamin.*

Sample No.	Description.	No. of pigeons used for test.	Duration of test.	Sample given on alternate days.	Average change in weight of pigeons.
			days	gm.	gm.
25-112	Autoclaved bread yeast.	2	8	1.0	-20
25-78	Yeast vitamin (Harris) Lot 999.	3	10	0.10	-51
		3	8	0.20	-16
		3	10	0.30	-37
25-78	" " " " 997.	3	10	0.20	-31
24-194	Dried brewers' yeast.	4	10	0.30	+19
		4	10	0.10	-9
24-195	" yeast protein.	5	10	0.30	-17
23-192	Activated solid.	6	10	0.20	+23
24-196(a)	" "	3	8	0.20	+12
		3	10	0.10	-2
G 6-7	" "	3	12	0.05	-5
		3	14	0.03	-17
G 11	" "	3	12	0.05	-13
		4	10	0.03	-17

ing to about 100 kilos of fresh yeast) there are obtained between 200 and 300 gm. of inactive organic material and approximately 200 gm. of the highly active product, about one-fourth of which is, however, not obtained as non-hygroscopic powder but remains dissolved in the final alcoholic solution. It is hoped that further work will reveal a method for also converting this last portion to the dry state.

<sup>6</sup> Seidell, A., *Public Health Rep., U. S. P. H.*, 1922, xxxvii, 1519.

TABLE II.

*Change in Weight of Pigeons Fed on Polished Rice and Given on Alternate Days the Specified Doses of the Several Fractions Obtained in the Process of Concentrating the Aqueous Extract of the Antineuritic Vitamin.*

Sample No.	Description.	No. of pigeons used for test.	Duration of test.	Sample given on alternate days.	Average change in weight of pigeons.
			days	gm.	gm.
25-151	Precipitate obtained from aqueous extract without addition of alcohol.	4	6	0.25	-30
25-85	Precipitate obtained in presence of about 30 per cent $\text{CH}_3\text{OH}$ .	2	10	0.10	-23
25-127	Precipitate obtained in presence of about 50 per cent $\text{CH}_3\text{OH}$ .	3	8	0.10	-14
25-159	Precipitate obtained in presence of about 20 per cent $\text{CH}_3\text{OH}$ .	3	8	0.10	-15
25-128	Precipitate obtained in presence of about 85 per cent $\text{CH}_3\text{OH}$ .	4	10	0.050	+16
		4	8	0.025	-6
		3	6	0.015	-2
25-96	Precipitate obtained by pouring a $\text{CH}_3\text{OH}$ solution of the viscous solids into 98.5 per cent $\text{C}_2\text{H}_5\text{OH}$ .	4	10	0.010	$\pm 0$
25-99	Dry powder obtained by treating viscous distillation residue with 98.5 per cent $\text{C}_2\text{H}_5\text{OH}$ .	3	8	0.020	+9
		4	10	0.020	-4
		4	10	0.010	-13
		4	8	0.010	-12
		4	8	0.006	-21
25-130	" "	3	8	0.020	+10
		3	8	0.010	-8
25-139	" "	4	10	0.020	$\pm 0$
		3	6	0.010	-10
25-162	" "	3	6	0.030	+20
		3	6	0.020	+14
		3	8	0.010	+13
		4	10	0.005	-8
25-167	" "	4	8	0.020	+20
		4	8	0.010	-5
25-105	Dissolved solids in final alcoholic solution.	4	8	0.020	-2
25-143	" "	4	8	0.022	-14
25-171	" "	2	10	0.020	-2

On the basis of units of activity, as determined roughly by pigeon feeding tests, approximately one-third of the total anti-neuritic vitamin contained in the 5 kilos of activated solid is found in about 150 gm. of non-hygroscopic powder and 50 gm. of solids contained in the final alcoholic solution. The losses of active material are undoubtedly due to (1) the impossibility of liberating all of the vitamin from its combination with fullers' earth; (2) to its destruction by the alkali and the various treatments to which its solutions are subjected during the process; and (3) to the impossibility of completely separating it from the inactive fraction by the present method.

Numerous tests of the non-hygroscopic active powder on pigeons have shown that it prevents loss in weight, or restores that resulting from an exclusive diet of polished rice, in doses of about 0.010 gm. given on alternate days. Comparative tests of several samples of the product and of the inactive fractions are shown in Tables I and II.

#### SUMMARY.

A relatively simple method for preparing fairly large quantities of a highly active non-hygroscopic vitamin concentrate from brewers' yeast is described. The several steps of the method are: removal of the yeast protein by boiling; adsorbing the vitamin from the resulting solution by means of fullers' earth; extracting the activated solid with sodium hydroxide; acidifying and concentrating by vacuum distillation; removing the sodium as  $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$  and the non-active organic material by means of methyl alcohol; finally, converting the active viscous residue to a non-hygroscopic dry powder by treatment with strong ethyl alcohol.

*Addendum.*—In a note which appeared since the above was written, Levene and van der Hoeven<sup>7</sup> announce an improvement of their process for the "Concentration of the Growth Promoting Principle Obtained from Yeast (Vitamin B)." It is stated that, starting with the Osborne and Wakeman fraction, there is obtained, by only three steps, a concentrated product, active for rats in doses of 0.0018 gm., and representing 50 per cent of the vitamin in the original material. In this connection it may be ob-

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<sup>7</sup> Levene, P. A., and van der Hoeven, B. J. C., *Science*, 1925, lxi, 594.



served that the Osborne and Wakeman concentrate is prepared by fractional precipitation with alcohol and does not contain all the vitamin originally present in the yeast. An estimate of the losses in the process is affected by the same uncertainties mentioned in connection with the separation of the active from the inactive fractions in the present paper. If it is assumed, however, that Osborne and Wakeman's active Fraction II contains two-thirds of the total vitamin of the yeast, and one-half of this is further concentrated by the new procedure of Levene and van der Hoeven, the final yield of active product is about the same as that obtained by the method here described.

In regard to the activity of vitamin fractions, as determined by feeding experiments, the results will in all cases be influenced by the technique employed. On the basis of experience gained in this laboratory by Doctor Smith, a 3 day period for judging the effect of a given sample upon rats, as practised by Levene and van der Hoeven, might be expected to lead frequently to erroneous conclusions. It has been found that in a large percentage of cases, doses of a given sample, which are insufficient to cause a continued increase in weight for 10 days or more, will show as much as a 5 to 7 gm. increase during the first 3 days of the period. Much smaller daily amounts of a sample will serve, in the majority of cases, to produce a 3 day increase in the weight of a rat than are sufficient for a longer period.

# THE DISTRIBUTION OF SOME OF THE MORE IMPORTANT AMINO ACIDS IN THE GLOBULIN OF THE THYROID GLAND.

BY HENRY C. ECKSTEIN.

*(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor.)*

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Thyreoglobulin, the principal protein of the thyroid gland, is peculiar inasmuch as it is one of the few native proteins containing iodine. Baumann's (1) discovery of this fact stimulated interest in this globulin and with the development of methods for protein analysis some knowledge of its amino acid make-up was obtained. Nürenberg (2), employing the method of Kossel and Kutscher (3), determined the percentages of the individual basic amino acids in thyreoglobulin and also demonstrated the presence of tyrosine and glutamic acid. Koch (4) likewise determined the bases by the Kossel and Kutscher method, but the results which he obtained were not completely in accordance with those reported by Nürenberg. The fact that thyreoglobulin has not been analyzed by means of the more recent methods for protein analysis suggested the advisability of such an investigation. The protein was consequently analyzed by the Van Slyke method (5). Further information was secured by determining tyrosine, tryptophane, and cystine according to the procedure outlined by Folin and Looney (6). The percentage of arginine was also determined by the very recent method of Kossel and Gross (7) and histidine by a procedure outlined by Plimmer and Phillips (8).

The material used for analysis was isolated from hog thyroid according to the methods outlined by Oswald (9). Two preparations were made. Preparation I was obtained by extracting the glands with physiological saline and precipitating the globulin from solution by adding an equal volume of a saturated solution of ammonium sulfate. Preparation II was likewise obtained by extracting the glands with physiological saline, but the protein was thrown out of this solution by the addition of small amounts of dilute acetic acid.

The resulting products were analyzed by the methods already outlined. Most of the results of these analyses are summarized

in the accompanying tables. A few additional ones are given in the text.

That a considerable difference exists between the results obtained by the writer and those reported by others is evident from a glance at Table I. This summarizes the author's results and also shows the results published by Nürnberg (2) as well as those obtained by Koch (4). A detailed report of the results secured by the writer from the Van Slyke analysis of Preparation I is given in Table II. It is quite evident that Koch as well as Nürnberg found much larger amounts of lysine in the protein than did the writer. It is also clear that the percentages of arginine and his-

TABLE I.

*Comparison of the Average Results of Preparations I and II with Those Reported by Others.*

Preparation .....	I	II	Koch (4).	Nürnberg (2).
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Arginine N.....	16.55	16.27	11.70	8.71
Histidine N.....	11.92	11.34	5.14	4.92
Lysine N.....	4.43	4.19	12.57	12.33
Cystine N.....	0.96	0.98		
Amino N in bases.....	13.51	13.12		
Amide N.....	6.52	6.76		5.24
Melanin N.....	1.56	1.62		
Amino N in filtrate.....	52.78	54.24		
Non-amino N in filtrate.....	4.49	5.13		
Total.....	99.21	100.53		

tidine which were published by both Koch and Nürnberg are lower than those found in this investigation. In Table III are grouped the results secured from the analysis of Preparation II. It is quite apparent that these data differ but little from those compiled in Table II. It follows, therefore, that so far as the Van Slyke analysis is concerned Preparations I and II are identical. The fact that the nitrogen as well as the iodine contents of both preparations agree well with the results reported by others makes it seem unlikely that the samples analyzed by the writer differ from those analyzed by others. Preparation I contained 15.62 per cent nitrogen and 1.60 per cent iodine, Preparation II, 15.55 per cent

nitrogen and 1.88 per cent iodine. The percentage of iodine in the protein was determined by the method of Kendall (10).

The question naturally arises whether the Van Slyke method is as reliable for determining the basic amino acids as is the Kossel and Kutscher method. The writer is of the opinion that it is, for the reason that arginine, histidine, lysine, and cystine determinations made with other methods agree well with those secured with the Van Slyke method.

That there is not as much as 12 per cent lysine nitrogen present in either Preparation I or II follows from the fact that the total amino nitrogen in the solution of the bases never exceeds 14.5 per

TABLE II.

*Results of Analysis of Preparation I in Percentages of Total Nitrogen Obtained by the Van Slyke Method.*

Sample.....	1	2	3	4	5	6
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Arginine N.....	17.41	16.93	16.84	15.71	16.23	16.21
Histidine N.....	12.72	12.45	11.91	10.92	11.45	12.10
Lysine N.....	4.71	5.23	4.12	3.92	4.21	4.42
Cystine N.....	0.83	0.72	0.81	1.01	1.21	1.16
Amino N in bases.....	13.94	14.23	13.11	12.83	13.30	13.66
Amide N.....	7.11	7.22	6.81	5.93	6.21	5.86
Melanin N.....	1.71	1.33	1.50	1.60	1.10	2.10
Amino N in filtrate.....	52.96	51.99	52.24	53.62	54.10	51.76
Non-amino N in filtrate.....	4.81	4.35	5.16	4.72	4.10	3.84
Total.....	102.26	100.22	99.39	97.43	99.61	97.45

cent. All of the nitrogen in the lysine molecule is in the form of amino nitrogen. If there should, therefore, be as much as 12 per cent lysine nitrogen in the globulin, there could be but little of the other basic amino acids present. That histidine and arginine are present in marked amounts is very evident from the numerous analyses which are summarized in Tables II and III. A separate determination of the percentage of the free amino acid nitrogen in the original protein revealed the presence of 1.9 per cent of nitrogen in this form. According to Van Slyke and Birchard (11) the percentage of free amino nitrogen in proteins is an index of the amount of lysine nitrogen present. The fact that one-half of the lysine nitrogen as determined by the Van Slyke method was 2.2 and 2.1

respectively for Preparations I and II is evidence substantiating this view of Van Slyke and Birchard. It also favors the contention of the writer that neither Preparation I nor II contains more than 5 per cent of lysine nitrogen.

That the Van Slyke method for arginine is reliable follows from the fact that the author obtained arginine figures for thyreoglobulin by means of the very recent method of Kossel and Gross (7) which agree exceedingly well with those secured with the Van Slyke method. In the new method of Kossel and Gross the arginine is precipitated by means of an aqueous solution of 2,4-dinitro-1-naphthol-7-sulfonic acid and the resulting arginine salt weighed.

TABLE III.

*Results of Analysis of Preparation III in Percentages of Total Nitrogen Obtained by the Van Slyke Method.*

Sample.....	7	8	9	10	11	12
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Arginine N.....	16.41	16.62	17.20	15.91	15.81	15.62
Histidine N.....	10.95	11.21	11.93	12.71	11.75	11.53
Lysine N.....	5.15	3.87	4.13	3.91	3.84	4.23
Cystine N.....	0.97	1.01	0.99	1.21	0.88	0.76
Amino N in bases.....	13.87	12.77	13.41	13.34	12.59	12.76
Amide N.....	7.10	6.83	7.31	5.96	6.25	7.10
Melanin N.....	2.11	1.76	1.52	1.77	1.19	1.37
Amino N in filtrate.....	54.90	55.61	54.95	54.26	53.31	54.47
Non-amino N in filtrate.....	4.81	5.86	4.12	5.32	4.96	5.7
Total.....	102.40	100.77	102.15	101.05	97.97	100.7

Various considerations made it appear advisable to use the solution of the bases obtained in the Van Slyke procedure for determining arginine by Kossel and Gross's method. The acidity of this solution proved to be ideal for the precipitation of the amino acid, and the absence of sodium, potassium, and calcium salts in this solution assured a desirable medium for precipitating arginine only. The percentages of arginine nitrogen as determined on 10 cc. portion of the solution of the bases from Samples 10, 11, and 12 were 11.1, 16.1, and 16.3 respectively. The low results obtained from Sample 10 were undoubtedly due to a failure to use water cooled to 0°C. in washing the arginine salt. The salts secured from Samples 11 and 12 were washed with water cooled to the above temperature and

these instances the percentages of arginine nitrogen agree exceedingly well with those found in the Van Slyke method. That the precipitates weighed were actually the arginine salts of the sulfonic acid and nothing else was conclusively established in the following manner. The precipitates obtained from Samples 10, 11, and 12 were combined, dissolved in a 5 per cent solution of sulfuric acid, and the resulting mixture extracted with cold normal butyl alcohol. According to Pratt (12) this procedure results in a separation of the dye from the arginine, the former passing into the alcohol layer, the latter remaining in the acid layer. This acid layer was quantitatively separated from the butyl alcohol solution, diluted to a known volume, and the percentage of arginine determined therein by boiling aliquots for 6 hours in the presence of sodium hydroxide. The ammonia nitrogen which was evolved as a result of this hydrolysis corresponded to the amount of nitrogen that should have been evolved in this form from the amount of arginine as determined gravimetrically. This demonstrates conclusively that the precipitates were precipitates of the arginine salt of the sulfonic acid and nothing else. There must therefore be as much as 16 per cent of arginine nitrogen in Preparations I and II.

Further favorable evidence supporting the view that the results obtained with the Van Slyke method are reliable was secured by showing that the amount of histidine in thyreoglobulin as determined by the bromination method of Plimmer and Phillips (8) agreed well with the results summarized in Tables II and III. The average of three determinations proved to be 11.5 per cent. This figure is in close agreement with average values given in Table I.

From the foregoing facts it is clear that the Van Slyke method gives results which are reliable. There can be no doubt that the method of Kossel and Kutscher also gives good results. At first sight no plausible explanation is at hand to explain the variations obtained by analyzing thyreoglobulin by the two methods. A study of Nürenberg's paper, however, reveals the following facts. The percentage of lysine was not determined by weighing the picrate of that acid as is recommended by Kossel and Kutscher, but by merely determining the total nitrogen of the lysine fraction. Histidine and arginine were likewise determined indirectly. If the

precipitation of arginine and histidine had been incomplete, then high lysine and low arginine and histidine values would have been obtained. Further examination of Nürenberg's data shows that a loss of nitrogen occurred during the separation of arginine from histidine. Such a loss would naturally result in a loss of the amino acids being determined. These facts may serve to explain the differences referred to.

Attention has already been called to Nürenberg's (2) demonstration of the presence of tyrosine in thyreoglobulin. He failed, however, to give any quantitative data. The writer, making use of the method of Folin and Looney (6), determined the percentage of tyrosine, tryptophane, and cystine in the protein and found 5.45 per cent of tyrosine, 2.15 per cent of tryptophane, and 1.55 per cent of cystine. Little comment is necessary on these results other than to point out that so far as these amino acids are concerned thyreoglobulin is not an unusual protein. The percentage of cystine when recalculated as the per cent of total nitrogen agrees well with results obtained with the Van Slyke method.

#### SUMMARY.

1. Thyreoglobulin analyzed by the Van Slyke method for protein analysis gave results for arginine, histidine, and lysine nitrogen which differed considerably from the results reported by workers using the Kossel and Kutscher method.

2. The percentage of arginine nitrogen as determined by the very recent method of Kossel and Gross and the percentage of histidine found by the bromination method of Plimmer and Phillips both agreed well with the percentages of arginine and histidine nitrogen determined by the Van Slyke method.

3. The percentage of free amino nitrogen in the original protein was found to equal practically one-half of the percentage of lysine nitrogen as determined by the Van Slyke method.

4. The percentages of tryptophane, tyrosine, and cystine were determined by the method of Folin and Looney. The amount of cystine obtained by this method agrees well with the amount of cystine found to be present by the Van Slyke method.

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## STROPHANTHIN.

### IX. ON CRYSTALLINE KOMBE STROPHANTHIN.

BY WALTER A. JACOBS AND ALEXANDER HOFFMANN.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

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A crystalline strophanthin which accompanies the so called amorphous strophanthin in alcoholic extracts of *Strophanthus kombe* has been observed by a number of investigators. This substance has been studied comparatively recently and almost simultaneously by Heffter and Sachs,<sup>1</sup> and by Brauns and Closson.<sup>2</sup> The excellent critical, historical review of the chemistry of the different strophanthins with which Heffter and Sachs have preceded their own report has accomplished the good service of placing a proper interpretation on a number of conflicting previous investigations which were performed at a time when botanical classification of the various *Strophanthus* plants was uncertain. From this review it appears that Arnaud<sup>3</sup> was the only other chemist who had previously made any study of the properties and composition of crystalline Kombe strophanthin, although before him Fraser and Catillon had probably already noted its occurrence. Arnaud prepared the substance by clearing an aqueous solution of the concentrated alcoholic extract of the seeds with basic lead acetate and by subsequent concentration to crystallization of the mother liquor from which excess lead had been removed. After several recrystallizations from water, sparingly soluble rosettes of crystals were obtained which softened at 165° and showed a composition of C, 60.46; H, 8.07; and  $[\alpha]_D = +30^\circ$

<sup>1</sup> Heffter, A., and Sachs, F., *Biochem. Z.*, 1912, xl, 83.

<sup>2</sup> Brauns, D. H., and Closson, O. E., *J. Am. Pharmaceut. Assn.*, 1913, ii, 715.

<sup>3</sup> Arnaud, M., *Compt. rend. Acad.*, 1888, cvii, 179.

in water. Thoms<sup>4</sup> later found that this sample gave a green coloration with sulfuric acid. Heffter and Sachs worked with commercial Kombe seeds of recognized botanical purity and employed a method essentially similar to that of Arnaud. They described their substance as neutral, and sparingly soluble, and one which crystallized in voluminous aggregates of fine, long, radially grouped needles. When dried, the substance softened to a paste at 177–181° and gave a deep green color with sulfuric acid (8:2). Their analytical results showed C, 61.93; H, 7.64; OCH<sub>3</sub>, 4.73; and  $[\alpha]_D^{20} = +28.72^\circ$  in water. On hydrolysis strophanthidin was produced and also a reducing sugar solution which could not be crystallized and from which no osazone could be obtained. Brauns and Closson employed carefully identified Kombe seeds and again used essentially the method of Arnaud. From their description, crystalline strophanthin consists of microscopic fine needles, or long plates which, when air-dried, contain 6 to 7 per cent of water and melt to a turbid mass at 158–165°, or at 178–179°, when air-dry or anhydrous respectively. Their analyses showed C, 61.97; H, 7.98; and  $[\alpha]_D^{20} = +28.7^\circ$  in water. In concentrated sulfuric acid a dark green color was first obtained which changed on standing to a brownish color.

In a preliminary note<sup>5</sup> from this laboratory, several years ago, we have described a crystalline strophanthin obtained from commercial Kombe seeds by an essentially similar method which agreed in properties very closely with the above descriptions. It formed sparingly soluble delicate needles, or platelets, which, when air-dried, melted at 180–183°. Analysis showed the presence of 7.6 per cent of water and C, 62.15; H, 7.55; OCH<sub>3</sub>, 5.26; and  $[\alpha]_D^{20} = +30.5^\circ$  in 95 per cent alcohol.

It is thus seen that the so called crystalline Kombe strophanthins which have all been obtained and purified by similar methods agree, except for minor variations, so closely in properties that there is little question that the same substance has been the subject of study in each case. The variations in melting points observed are not significant, and such variations are of no importance in view of what follows.

Following our preliminary note on crystalline Kombe strophanthin, we have gradually accumulated more material with the inten-

<sup>4</sup>Thoms, H., *Ber. pharm. Ges.*, 1904, xiv, 112.

<sup>5</sup>Jacobs, W. A., *J. Biol. Chem.*, 1923, lvii, 569.

tion of giving more thorough study to the carbohydrate moiety of the molecule which we had found to belong, in all likelihood, to the group of desoxy sugars and which gave a purple reaction in acetic acid with iron and sulfuric acid. Further work has given a rather surprising turn to these studies, since it has now been found that the so called crystalline Kombe strophanthin is, in reality, a mixture. In the dry state this glucoside is but very sparingly soluble in chloroform and forms a pasty mass under this solvent. If, however, water is added to the mixture, the pasty mass dissolves and investigation showed that this operation separated the substance into a chloroform-soluble and a water-soluble portion. On working up the chloroform extract by concentration and precipitation with ligroin, a copious amorphous precipitate was formed in a yield of about 50 per cent of the starting material. This was found to crystallize beautifully from methyl alcohol forming long prisms which melted at  $148^{\circ}$ . From dilute alcohol the substance could be obtained in another crystalline form which melted at  $185-187^{\circ}$ . Analysis of the anhydrous substance gave C, 65.9; H, 8.07; and  $\text{OCH}_3$ , 5.73. In alcoholic solution  $[\alpha]_D = +37.5^{\circ}$ . By the Keller-Kiliani reaction, the substance yielded the deep blue color which has been described by Kiliani<sup>6</sup> as characteristic for digitoxose and which was also observed in the case of cymarín and cymarose by Windaus and Hermanns.<sup>7</sup> The analytical figures, color reactions, and other properties of this substance were such as to suggest its identity with cymarín, the crystalline glucoside first isolated by Taub and Fickewirth of the Farbenfabriken vorm. Friedr. Bayer and Co. from various species of *Apocynum*, and which Windaus and Hermanns have demonstrated to be a glucoside of strophanthidin and cymarose, the latter possibly a methyl ether of digitoxose. Since in a few minor points (melting points and rotation) our observations differed from the properties recorded by the latter workers, cymarín was prepared directly from Canadian hemp for comparison. This comparison established definitely the identity of the chloroform-soluble portion of "crystalline Kombe strophanthin" with the cymarín of Canadian hemp. The Kombe "cymarín" yielded not only strophanthidin, but the cymarose

<sup>6</sup> Kiliani, H., *Arch. Pharm.*, 1896, cexxiv, 275.

<sup>7</sup> Windaus, A., and Hermanns, L., *Ber. chem. Ges.*, 1915, xlviii, 979.

described by these workers. It is thus seen that the intimate relationship of the cardiac poison of *Apocynum* with that of *Strophanthus kombe* also of the family Apocynaceæ, already demonstrated by the identification of the strophanthidins obtained from both sources, is made more striking by the demonstration of the common occurrence of the identical glucoside in each of these groups of plants.

An investigation of the water-soluble portion of crystalline K-strophanthin, after separation of the chloroform fraction, has shown it to be still a mixture of substances. On concentration, about 13 per cent of a second crystalline glucoside was obtained which proved to be a neutral substance, sparingly soluble in water, which melted with effervescence at 150–151°, and gave a pure, emerald green color with sulfuric acid (4:1), untinged with brown. On analysis, it gave figures which agreed with the formula  $C_{38}H_{54}O_{14}$  and showed the presence of one methoxyl group. On gentle hydrolysis it yielded strophanthidin and a strongly reducing sugar solution which, however, on further hydrolysis, was enhanced in reducing power by about 70 per cent. The sugar in the original glucoside, therefore, is a biose, probably with the formula  $C_{12}H_{24}O_9$ , and since it contains one methoxyl, this would agree with a biose consisting of a hexose and cymarose. With the material available, it has not been possible to make a definite decision in this regard, but our conclusions, we believe, are supported by the preparation of a crystalline tetracetyl derivative of the new crystalline strophanthin. No crystalline osazone could be obtained from the completely hydrolyzed sugar solution. In its color reactions, the new glucoside has failed to give a certain indication of the presence of cymarose. The Keller-Kiliani test was practically negative, but when the test was performed as given by one of us, and under conditions which, with the original glucoside, gave a purple color, and with cymarin a deep green color, the new glucoside developed, at first, a green, followed by a purple; but the color tones were not as clear as in the case of the former substances. The presence of cymarose in this glucoside is suggested by these reactions and the failure of the Keller-Kiliani test may be due to the character of the union with the other sugar which can be broken only under conditions which cause decomposition of the unstable desoxy sugar.

On distillation with hydrochloric acid, no furfural or methylfurfural was obtained from the glucoside, so that pentoses and methyl pentoses are excluded. A similar observation was made by Brauns and Closson in their examination of the so called crystalline K-strophanthin for rhamnose, which led them to question the results of Feist,<sup>8</sup> who had previously worked with a supposedly crystalline Kombe strophanthin, from which a so called strophanthobiose methyl ether was isolated. This sugar Feist concluded to be a methyl ether of a mannose-rhamnose biose. As already discussed by Heffter and Sachs, it is greatly to be doubted that Feist, in reality, worked with a crystalline substance. Feist stated that it was prepared by Fraser's method, in accordance with which the purified alcoholic solution of the crude glucosides obtained from the seeds was precipitated by ether. Such a method should eliminate most of the cymarins, since this is soluble in such an alcohol-ether mixture. We are convinced that the results of Feist were obtained with a mixture of amorphous glucosides and have no bearing on the question of "crystalline Kombe strophanthin." We are continuing the study of the sugars in the new glucoside. As a continuation of the suggestion of Thoms<sup>4</sup> for the nomenclature of the strophanthins, we propose the designation K-strophanthin- $\beta$  for this new strophanthidin bioside, since, in all likelihood, still others may be found. Although, in accordance with this scheme, it would be logical to call cymarins K-strophanthin- $\alpha$ , the former name has already been introduced and there appears no reason to change it to the less wieldy designation.

The aqueous mother liquor from K-strophanthin- $\beta$  yielded an easily soluble, amorphous substance which was, apparently, a mixture of a number of glucosides and which gave reactions identical with those given by the former.

Since our experiments were performed with commercial Kombe seeds which might have caused uncertainty, we have been extremely fortunate to receive from Parke, Davis and Company a sample of crystalline K-strophanthin in answer to our request for some of the material prepared by Brauns and Closson from identified seed. The examination of this material confirmed in all respects the results obtained with the substances of our own

<sup>8</sup> Feist, F., *Ber. chem. Ges.*, 1898, xxxi, 534; 1900, xxxiii, 2069.

preparation with the exception that the proportions of crystalline glucosides obtained differed somewhat. It is questionable whether "crystalline *Kombe strophanthin*" represents a constant mixture and it is probable that the substances studied by the various workers have been of somewhat different composition.

From these experiments it is obvious that extracts of *Strophanthus kombe* seeds contain a mixture of strophanthidin glucosides and it appears that of all the strophanthins from this source which have been examined, either chemically or pharmacologically in the past, in no case can there be certainty that a homogeneous substance has been employed. In their very careful investigations with identified *Strophanthus hispidus* seeds, Heffter and Sachs were unable to obtain evidence of the presence of a crystalline glucoside, so that it is doubtful whether cymarín can be isolated from this source, or at least only in negligible amounts. It is very likely that their amorphous *hispidus* strophanthin was also a mixture. Cymarín and K-strophanthin- $\beta$  are the only glucosides of strophanthidin at present known which we can be certain are chemical individuals. It is, therefore, of importance to make further attempts to isolate as far as this is possible other individual glucosides which occur in these plants and to submit them to pharmacological study.

Finally, the preparation of a monoacetyl cymarín from cymarín in pyridine solution with acetic anhydride is of interest in suggesting the mode of linkage of the sugar on strophanthidin. If we accept what is most likely, that acetylation has occurred on the free hydroxyl of cymarose, then strophanthidin has remained unacetylated. Since strophanthidin itself forms only a monoacyl compound under such conditions, the conclusion appears warranted that the hydroxyl<sup>9</sup> in strophanthidin which is ordinarily acylatable and which is presumably  $\gamma$ - to the aldehyde group is here the point of glucosidic union with the sugar, cymarose.

#### EXPERIMENTAL.

The so called crystalline K-strophanthin employed in the following experiments was prepared by the method<sup>4</sup> previously described

<sup>9</sup> Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1922, liv, 253.  
Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lix, 718.

from commercial Kombe seeds which gave uniformly a green color with sulfuric acid. The well washed crude crystalline glucoside as first obtained by the careful addition of ammonium sulfate to the aqueous solution of the crude glucoside mixture was recrystallized once as previously given. For further recrystallization we have adopted a somewhat different procedure.

10 gm. of the glucoside were dissolved in 6 cc. of warm 95 per cent alcohol and the solution was then treated with 50 cc. of water. The clear solution on standing gradually deposited needles which possessed the properties already recorded.

$$[\alpha]_D = +30.2^\circ \text{ (c = 1.000 in 95 per cent alcohol).}$$

For the substance dried at  $100^\circ$  at 20 mm. over  $\text{H}_2\text{SO}_4$ .

Found. C 62.52, H 7.88.

This substance when treated according to Heffter and Sachs<sup>1</sup> with sulfuric acid which was diluted with one-quarter volume of water developed a deep brownish green color.

A small amount of substance when dissolved in acetic acid and then treated with a few crystals of ferrous sulfate followed by a few drops of sulfuric acid produced a deep purple color as previously described. If, however, the Keller-Kiliani reaction was performed as described by Kiliani,<sup>6</sup> a slowly developing characteristic deep blue color was obtained due to the presence of cymar in the glucoside.

#### *Preparation of Cymar in from Crystalline K-Strophanthin.*

20 gm. of crystalline K-strophanthin were shaken several hours in a mixture of 200 cc. of chloroform and 200 cc. of water. Although the starting material is apparently very little soluble in chloroform alone, by this procedure almost complete solution occurred. After separation of the layers the aqueous portion was extracted several times with small additional portions of chloroform. The united chloroform extracts were shaken again with a little water, dried over  $\text{CaCl}_2$  and then concentrated to 50 cc. On addition of 700 cc. of petroleic ether a flocculent precipitate formed which after standing at  $0^\circ$  was collected. The dry substance weighed 11 gm. For further purification the crude cymar was dissolved in 25 cc. of hot methyl alcohol and the solution



was treated with 100 cc. of water and recrystallized in long well formed crystals which were collected with cold water. The yield of the air-dry substance was 7.5 gm. The mother liquor yielded an additional 2.5 gm. of crystalline material after removal of the methyl alcohol.

From aqueous methyl alcohol the substance formed long prisms which sintered on rapid heating at  $138^\circ$  and melted with effervescence at  $148^\circ$  (Windaus and Hermanns give  $130\text{--}138^\circ$ ). Cymarine prepared from Canadian hemp and recrystallized under the same conditions melted at the same point as did also a mixture of the two. The substance obtained by the above method of recrystallization separates apparently with 1 mol of methyl alcohol which is partly lost when the substance is air-dried since the loss on drying for analysis as well as the methoxyl determinations gave figures which were somewhat lower than those required by the theory.

*Air-Dry Substance.* Dried at  $78^\circ$  at 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{30}\text{H}_{44}\text{O}_8 \cdot \text{CH}_3\text{OH}$ . Calculated.  $\text{CH}_3\text{OH}$  5.52. Found,  $\text{CH}_3\text{OH}$  4.40.  
Zeisel. "  $2(\text{OCH}_3)$  10.69. "  $2(\text{OCH}_3)$  8.95.

*Anhydrous Substance.*

$\text{C}_{30}\text{H}_{44}\text{O}_8$ . Calculated. C 65.66, H 8.09,  $\text{OCH}_3$  5.65.  
Found. " 65.90, " 8.07, " 5.73.

When recrystallized from dilute ethyl alcohol the substance formed hexagonal leaflets which contained 1.5 mols of water and melted at  $185\text{--}187^\circ$  after slight preliminary sintering.

*Air-Dry Substance.* Dried at  $78^\circ$  and 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{30}\text{H}_{44}\text{O}_8 \cdot 1.5\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  4.93. Found.  $\text{H}_2\text{O}$  4.59.  
"  $\text{OCH}_3$  5.39. "  $\text{OCH}_3$  5.23.

Occasionally by this method of recrystallization a practically anhydrous cymarine was obtained which melted at  $204\text{--}205^\circ$ .

*For the Anhydrous Substance.*

$[\alpha]_D^{25} = +37.8^\circ$  (c = 4.94 in chloroform).

$[\alpha]_D^{25} = +37.5^\circ$  (c = 1.76 in 95 per cent alcohol).

For comparison the following observations were made with anhydrous cymarine prepared from Canadian hemp.

$[\alpha]_D^{25} = +35.0$  (c = 4.80 in chloroform).

$[\alpha]_D^{25} = +34.9$  (c = 1.32 in 95 per cent alcohol).

In its color reactions the cymarin from *K-strophanthin* proved identical with that from Canadian hemp. It gave the typical deep blue color with the Keller-Kiliani reagent but with the test as previously used by Jacobs for *K-strophanthin* only a dark green color was obtained with no suggestion of a purple. In sulfuric acid diluted with one-quarter volume of water only a brown color was obtained and no trace of a green color such as given by the starting material could be observed.

For further identification we have hydrolyzed the glucoside by the method given by Windaus and Hermanns. 7 gm. of cymarin yielded 5.1 gm. of strophanthidin or 97 per cent of the theory. This was characterized by the melting point  $172^{\circ}$ , rotation  $[\alpha]_D^{25} = +41.9$  ( $c = 1.98$  in methyl alcohol), and analysis,  $C_{22}H_{32}O_6 \cdot \frac{1}{2}H_2O$ .

Calculated. C 66.79, H 8.05.

Found. " 66.62, " 8.05.

From the aqueous mother liquor of strophanthidin the sugar, cymarose, was obtained as given by Windaus and Hermanns and was found to agree in all properties with the description of these workers. 1.2 gm. of needles were obtained which melted after recrystallization from anhydrous ether, petroleic ether at  $91^{\circ}$  ( $88^{\circ}$  Windaus and Hermanns).

$C_7H_{14}O_4$ . Calculated. C 51.81, H 8.70.

Found. " 52.02, " 8.59.

Cymarose shows in aqueous solution a slight mutarotation. For the final reading  $[\alpha]_D^{25} = +53.4^{\circ}$  ( $c = 2.245$  in  $H_2O$ ).

*Acetylcymarin*.—0.5 gm. of anhydrous cymarin was dissolved in 3 cc. of dry pyridine and the solution was treated with 1 cc. of acetic anhydride. The next day on dilution with water a milky emulsion was formed from which the acetyl compound slowly separated as rosettes. On dilution of the solution in methyl alcohol acetylcymarin forms silky needles which melted at  $160$ – $161^{\circ}$ , depending somewhat on the rate of heating. An acetylcymarin prepared from hemp cymarin showed the same properties.

0.1234 gm. of substance was refluxed for 1 hour in 15 cc. of alcohol and 14.1 cc. of 0.1 N NaOH and the mixture was titrated back against phenolphthalein. Calculated for two equivalents for monoacetyl cymarin, 4.15 cc. Found, 4.05 cc. Since one.

equivalent is required for the lactone group of strophanthidin the substance is a monoacetyl compound and the acetyl group must be situated on the one free hydroxyl of cymarose.

*Air-Dry Substance.* Dried at 100° and 15 mm. over H<sub>2</sub>SO<sub>4</sub>.

C<sub>32</sub>H<sub>46</sub>O<sub>10</sub> ·  $\frac{1}{2}$  H<sub>2</sub>O. Calculated, H<sub>2</sub>O 1.53. Found, 1.57.

*Anhydrous Substance.*

C<sub>32</sub>H<sub>46</sub>O<sub>10</sub>. Calculated. C 65.04, H 7.85.

Found. " 65.15, " 7.78.

### *The "Water-Soluble" Glucosides.*

*K-Strophanthin-β.*—The aqueous fractions obtained by the treatment of crystalline K-strophanthin with water and chloroform were united and concentrated under diminished pressure below 40° to small bulk, when the contents of the flask became a uniform crystalline mass which under the microscope appeared as long, thin, silky needles. These were collected with small portions of water and then carefully washed with cold water in which it appeared now to be but very sparingly soluble. The yield was 2.65 gm. It was found very difficult to recrystallize the substance.<sup>10</sup> It slowly dissolved on shaking in about 100 parts of water at ordinary temperature but on warming but little difference was noted in its solubility. However, the addition of small amounts of alcohol greatly increased the solubility but on cooling the substance did not separate again.

The new crystalline glucoside is neutral to litmus and melted, when air-dried, at 150–151° with effervescence. It is easily soluble in ethyl and methyl alcohol and practically insoluble in chloroform, acetic ether, ether, and petroleic ether. With sulfuric acid diluted with one-quarter volume of water it gives a pure emerald green color. The Keller-Kiliani reaction was negative but if the test is performed as described by Jacobs, under which conditions K-strophanthin gives a deep purple color, this glucoside gives at first a dirty green which changes to a violet. When distilled with hydrochloric acid, as in the determination of furfural and methyl furfural, the distillate gave no precipitate

<sup>10</sup> More recently the substance has been recrystallized by dissolving in two volumes of alcohol, by adding an equal volume of water and then by removing the alcohol under diminished pressure. When obtained under these conditions the substance showed a H<sub>2</sub>O content of 1.65 and melted at 176° after preliminary sintering.

with phloroglucin which should definitely exclude pentoses or methyl pentoses. Rotation for the anhydrous substance:

$$[\alpha]_D^{25} = +33.6^\circ \text{ (c = 0.97 in water).}$$

*Air-Dry Substance.* Dried at  $78^\circ$  and 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{30}\text{H}_{44}\text{O}_{14} \cdot 2\frac{1}{2}\text{H}_2\text{O}$ . Calculated,  $\text{H}_2\text{O}$  5.96. Found,  $\text{H}_2\text{O}$  5.92.

*Anhydrous Substance.*

$\text{C}_{30}\text{H}_{44}\text{O}_{14}$ . Calculated. C 60.81, H 7.66.

Found. " 60.66, " 7.62.

1 gm. of the crystalline glucoside was dissolved in a mixture of 8 cc. of 50 per cent alcohol and 2 cc. of concentrated hydrochloric acid, and allowed to stand for 6 hours, at  $20^\circ$ . On dilution, strophanthidin crystallized and additional amounts were obtained after removal of Cl ions with  $\text{Ag}_2\text{CO}_3$  and concentration of the mother liquor. 0.4 gm. was obtained, which possessed the usual properties. The mother liquor was concentrated under diminished pressure and finally, in a desiccator, to dryness. The resulting syrup showed no tendency to crystallize, but proved to be mostly a disaccharide, since, when a portion of the syrup was warmed in the water bath, for 15 minutes, with 2 per cent HCl, its reducing power for Fehling's solution increased by 70 per cent. Up to the present, with the limited material available, it has not been possible to identify the sugars. Rhamnose is certainly not present. The Keller-Kiliani test was practically negative. When boiled with strong hydrochloric acid, a yellow coloration is obtained, followed by the rapid deposition of brown flocks. After further hydrolysis, it did not yield a crystalline osazone. With orcinol and  $\alpha$ -naphthol it gave color reactions identical with those given by cymarose and in all likelihood the sugar is a biose of cymarose and a hexose.

*Tetracetate of K-Strophanthin- $\beta$ .*—0.5 gm. of the anhydrous glucoside was acetylated in pyridine solution with acetic anhydride. On dilution with water, 0.7 gm. of insoluble substance was obtained which was recrystallized by careful dilution of the alcoholic solution. It formed thin, microscopic needles which melted at about  $167^\circ$  to a vitreous mass. Titration after saponification gave figures in agreement with those required for four acetyl groups.

0.1 gm. was refluxed in 15 cc. of alcohol and 14.2 cc. of 0.1 N NaOH and titrated against phenolphthalein. Calculated for five equivalents (four acetyl and one lactone groups), 5.69 cc. Found, 5.55 cc.

*Air-Dry Substance.* Dried at 78° and 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{44}\text{H}_{52}\text{O}_{18} \cdot \text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  2.01.

Found. " 1.55.

*Anhydrous Substance.*

$\text{C}_{44}\text{H}_{52}\text{O}_{18}$ . Calculated. C 60.10, H 7.12.

Found. " 59.69, " 6.96.

*Water-Soluble Amorphous Fraction.*

The aqueous mother liquor from K-strophanthin- $\beta$ , when allowed to evaporate at ordinary temperature, yielded a non-crystalline, glassy residue, which weighed 5.35 gm. Although this substance was, in all probability, a mixture of glucosides, we shall record the few observations which were made. The melting point depended upon the rate of heating and varied from 160 to 170°. It was very soluble in water and alcohol, and insoluble in chloroform and acetic ether. The color reactions were the same as those given by K-strophanthin- $\beta$ .

$[\alpha]_D^{25} = +20.4^\circ$  ( $c = 5.27$  in water for the anhydrous substance).

Anhydrous substance (dried at 100° and 15 mm. over  $\text{H}_2\text{SO}_4$ ). C 59.07, H 7.39.

*Crystalline K-Strophanthin (Parke, Davis and Company).*

A sample of crystalline K-strophanthin, which was generously sent to us by Parke, Davis and Company in answer to our request for a sample of the material prepared in their laboratories by Brauns and Closson, from identified *Strophanthus kombe* seeds, was submitted to similar study. This distinctly microcrystalline substance sintered at 157° and melted at 177–179°, and gave the usual reactions for crystalline K-strophanthin. When 1 gm. was submitted to the treatment with water and chloroform, as previously described, 0.32 gm. of cymarín, 0.27 of K-strophanthin- $\beta$ , and 0.36 gm. of amorphous glucoside were obtained. The cymarín possessed the usual properties and melted at 148°.

The sample of K-strophanthin- $\beta$  melted at 154–156° with effervescence, and agreed in all other properties with our K-strophanthin- $\beta$ .

$[\alpha]_D^{25} = +32.7^\circ$  ( $c = 0.67$  in water for the anhydrous substance).

*Air-Dry Substance.* Dried at 78° and 15 mm. over  $\text{H}_2\text{SO}_4$ .

$\text{C}_{38}\text{H}_{44}\text{O}_{14} \cdot 2\text{H}_2\text{O}$ . Calculated.  $\text{H}_2\text{O}$  4.82.

Found. " 4.31.

*Anhydrous Substance.*

$\text{C}_{38}\text{H}_{44}\text{O}_{14}$ . Calculated. C 60.81, H 7.66.

Found. " 60.71, " 7.54.

# A PROCEDURE FOR THE DETERMINATION OF UREA IN FOLIN-WU BLOOD FILTRATES BY THE AUTOCLAVE METHOD.

By E. P. CLARK AND J. B. COLLIP.

(From the Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada.)

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The procedure herewith described for the determination of urea in Folin-Wu blood filtrates was developed to meet certain requirements in connection with some problems under investigation in this laboratory. In reporting on this procedure our main object is to record the data we have obtained upon the urea content of normal Folin-Wu blood filtrates by the autoclave method and also to call attention to the convenience of evolving and estimating the ammonia thus obtained by the Pregl technique in conjunction with a modified Parnas-Wagner micro Kjeldahl apparatus which will be described.

In the method we have adopted, a combination of three well known operations was utilized to give a system by which the urea in Folin-Wu blood filtrates could be estimated with a fair degree of accuracy. The first procedure used was that of converting the urea into ammonia by acid hydrolysis as suggested by Folin and Wu (1); the second consisted of evolving the ammonia thus formed by means of the micro Kjeldahl apparatus of Parnas and Wagner (2); and finally the ammonia was estimated by the Pregl technique (3). Urea is quantitatively converted into ammonia by acid hydrolysis at 150° and, as will be shown in the experimental part, the ammonia thus formed can be evolved by the Parnas-Wagner apparatus and collected in 0.01 normal acid and then estimated by the Pregl technique with the accuracy customarily realized by this system of microorganic analysis. It will be shown also that, within the limit of experimental error, the values obtained on Folin-Wu blood filtrates are due to urea

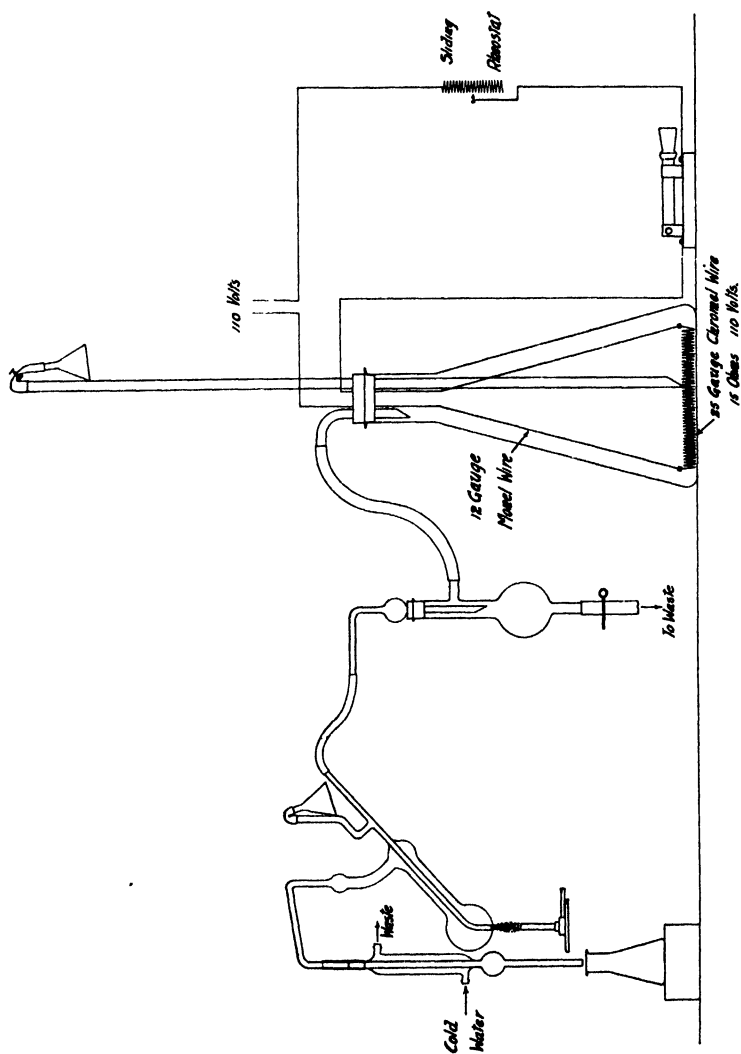


Fig. 1. Modified Parnas-Wagner micro Kjeldahl apparatus. Scale one-eighth full size

and ammonia. However, upon the basis of the generally accepted data for the ammonia concentration of normal blood, this constituent is found to be present in such minute quantities that it comes within the range of experimental error.

#### EXPERIMENTAL.

##### *Description of Apparatus.*

The Parnas-Wagner micro Kjeldahl apparatus which we have used is a somewhat modified form of the original. The entire instrument was made of two pieces of Pyrex glass, shown diagrammatically in the accompanying drawing (Fig. 1). This tends to give more compactness and necessitates only one connection.

TABLE I.  
*Calculated  $C_8H_9ON$ , N 10.37 Per Cent.*

Solution I. Amount taken for analysis 4.822 mg.		Solution II. Amount taken for analysis 5.0 mg.	
Acid required.	Nitrogen found.	Acid required.	Nitrogen found.
<i>cc. 0.01 N</i>	<i>per cent</i>	<i>cc. 0.01 N</i>	<i>per cent</i>
3.59	10.40	3.69	10.33
3.57	10.36	3.70	10.36
3.57	10.36	3.68	10.30
3.58	10.39	3.70	10.36
Average.....10.38		Average.....10.34	

The steam generator is electrically operated thus giving a more elegant and refined control. It consists of a 4 liter Erlenmeyer flask in which a coil of No. 25 chromel or nichrome wire having a resistance of 15 ohms is immersed in distilled water. The regulation of the desired amount of steam is obtained by an outside rheostat. In using this apparatus an objection raised by Pregl, that an error is caused due to the glass condenser tube, must be met. As far as we were able to discover Pyrex glass seems to be perfectly satisfactory for this purpose as will be shown by the following experiments in which the nitrogen in acetanilide was determined by the use of the apparatus.

Samples of 5 cc. each of two different solutions containing the amount of purified acetanilide indicated in Table I were taken for analysis.



Likewise the ammonia obtained from 1 mg. of urea by the procedure which follows gave no indication of an error due to the glass condenser. 5 cc. of an accurately prepared solution of purified urea containing 0.2 mg. of the base per cc. were placed in a lipped test-tube 15 by 120 mm. 1 cc. of normal HCl was added, the tube was covered with tin-foil, and autoclaved at 150° for 10 minutes. After removing the tube from the autoclave its contents were transferred to the Kjeldahl apparatus, the solution made alkaline with 3 cc. of normal NaOH, and distilled. The ammonia was collected in 0.01 normal HCl and the excess acid titrated with 0.01 normal NaOH using methyl red as an indicator, following strictly Pregl's technique. In transferring

TABLE II.  
*Calculated for  $\text{CH}_4\text{ON}_2$ , N 46.66 Per Cent.*

Solution I.		Solution II.	
Acid used.	Nitrogen found in urea.	Acid used.	Nitrogen found in urea.
<i>cc. 0.01 N</i>	<i>per cent</i>	<i>cc. 0.01 N</i>	<i>per cent</i>
3.34	46.76	3.32	46.48
3.33	46.62	3.33	46.62
3.33	46.62	3.33	46.62
3.33	46.62	3.32	46.48
3.34	46.76	3.33	46.62
Average.....46.67		Average.....46.56	

the liquid from the container in which it was autoclaved to the Kjeldahl apparatus it was found expedient and desirable to apply a thin film of vaseline to the lip of the test-tube.

The results of ten determinations using 1 mg. of urea in each are shown in Table II.

#### *Determination of Urea in Folin-Wu Blood Filtrates.*

5 cc. of the Folin-Wu blood filtrate were treated in exactly the same manner as given for the pure urea solution above. The number of cc. of 0.01 normal acid used multiplied by 28 gives the number of mg. of urea per 100 cc. of blood.

In order to prove that the results obtained were due to urea (and ammonia) alone the following procedure was employed:

1. Urea in the blood filtrate was determined by the above method.

2. The non-protein nitrogen was determined on the same filtrate using the Pregl method.

3. The total nitrogen was determined upon the blood filtrate after the urea had been removed as dioxanthylurea. This was accomplished by adding 5 cc. of glacial acetic acid to 5 cc. of blood filtrate, followed by the addition of 1 cc. of a freshly prepared 10 per cent methyl alcoholic solution of xanthhydrol. After allowing the mixture to stand for 2 hours the dioxanthylurea was carefully collected in a Pregl filtering tube, the filtrate and washings being caught in a micro Kjeldahl flask in which the combustion was carried out.

TABLE III.

Non-protein nitrogen.		Urea nitrogen.		Blank on reagents used to precipitate urea.	Non-protein nitrogen after removal of urea as dioxanthylurea.		
Acid used.	N per 100 cc. blood.	Acid used.	N per 100 cc. blood.		Acid used.	Acid used less blank.	N per 100 cc. blood.
<i>cc. 0.01 N</i>	<i>mg.</i>	<i>cc. 0.01 N</i>	<i>mg.</i>		<i>cc. 0.01 N</i>	<i>cc. 0.01 N</i>	<i>mg.</i>
1.39	38.92	0.69	19.32	0.18 cc. of N/100 acid.	0.91	0.73	20.44
1.38	38.64	0.69	19.32		0.90	0.72	20.16
1.38	38.64	0.69	19.32		0.89	0.71	19.88
1.38	38.72	0.69	19.32		0.90	0.72	20.16

4. A blank was run upon all the reagents used in the last step, care being taken to have the same concentrations, volumes, etc., as used in the actual experiment. The data upon three such determinations, using 5 cc. of blood filtrate in each test are given in Table III.

It is therefore evident that the urea plus the non-protein nitrogen, after the removal of urea as dioxanthylurea, is 39.48 mg. per 100 cc. of blood, whereas the non-protein nitrogen on the original filtrate is 38.72 mg. per 100 cc. of blood, thus making a difference of +0.76 mg. per 100 cc. or +1.9 per cent. This positive difference includes the dioxanthylurea dissolved in the

mother liquor from which it was precipitated and also the ammonia present in the blood. These factors would naturally reduce the error considerably, but for the purpose at hand it seems safe to conclude that the method is accurate to within less than 2 per cent in the case of normal blood. It is of course obvious that the per cent error would be proportionally less the higher are the values for urea and non-protein nitrogen.

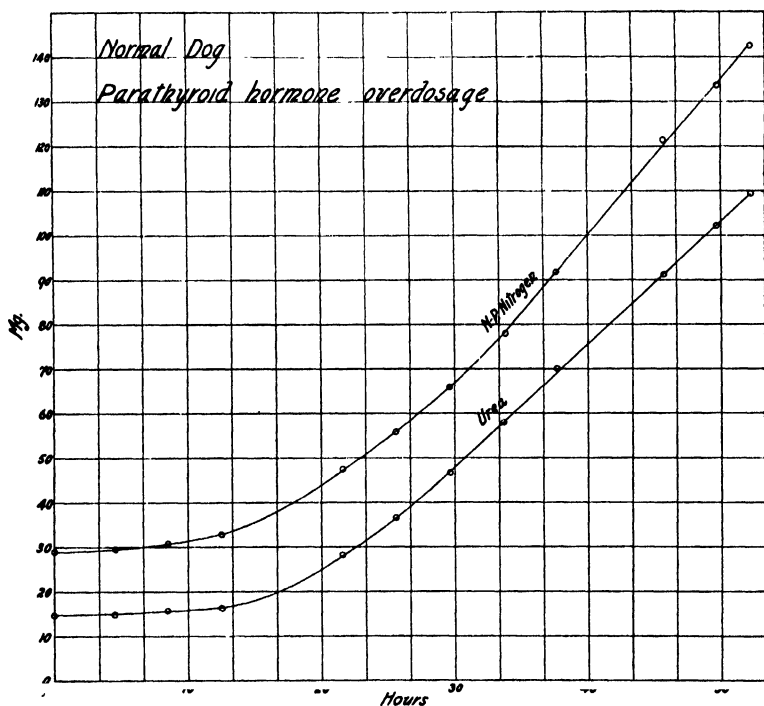


CHART I. Dog 331. ♂ 18.25 kilos. Blood urea and non-protein nitrogen curves showing the overdosage effect of the parathyroid hormone. Doses of 20 units were given at the same time blood samples were taken for analysis.

In order to show the possibilities of this method the accompanying graphs are given (Chart I). The curves show the overdosage effect of the parathyroid hormone upon a normal dog. Doses of 20 units were given at regular intervals so as to cause a pyramiding effect until death ensued.

## SUMMARY.

1. A combination of the autoclave method of Folin and Wu for converting urea into ammonia and the estimation of the ammonia thus formed, by the technique of Pregl and the apparatus of Parnas and Wagner, are utilized in developing an accurate chemical system for the determination of urea in Folin-Wu blood filtrates.

2. The autoclave method of Folin and Wu for the determination of urea in blood filtrates has been proved to give only urea (and ammonia) nitrogen.

3. An electrically controlled modification of the Parnas-Wagner micro Kjeldahl apparatus is given.

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## BLOOD SUGAR STUDIES.

By MAX GILBERT, HANS SCHNEIDER, AND JOSEPH C. BOCK.

*(From the Department of Physiological Chemistry, Marquette University, Medical School, Milwaukee.)*

(Received for publication, December 17, 1925.)

### INTRODUCTION.

The study of postprandial hyperglycemia is of steadily increasing importance. The usual methods for the determination of blood sugar necessitate a venous puncture in order to obtain a sample large enough for an accurate determination. This in turn usually means a longer time interval between the samples taken.

An analytical procedure has been recently perfected in this laboratory (4) which allows an accurate estimation of blood sugar in minute amounts of blood without resorting to venous puncture. It was thought to be of interest to use this method in the study of blood sugar time curves in which the time intervals are considerably shortened and the number of determinations increased, thereby obtaining more points of support and hence more accurate indications of any possible deviation from the accepted norm.

The subjects used in the experiments were with one exception (N. B. K.) apparently healthy young men of normal weight and build. Their clinical history gave no evidence of any previous illness of consequence. None of them showed glycosuria.

In the experiment, the subject was directed to come to the laboratory without breakfast. The majority of experiments, in fact, were started at noon so that the subjects had their last meal at least 16 hours before the first blood sample was taken. The amount of dextrose to be ingested was determined by multiplying the body weight expressed in kilos by 1.75. The sugar was dissolved in about 400 ml. of water. In some experiments ice water was used, in others the solution was warmed to room and body temperature respectively. In a few cases a small amount of citric acid was added as a flavoring.

Blood samples were drawn at various short intervals, making from fifteen to twenty-two samples in a period of approximately 3 hours. One or two samples were taken before giving the dextrose, and one sample at the moment the subject started drinking the solution. In Charts 1 to 26 the zero always designates the time when the sugar was taken.

In three cases about the same number of blood samples were drawn from individuals that had not ingested any sugar. One took 400 ml. of distilled water, the other two nothing at all. In all cases a specimen of the urine was collected and tested for sugar with Benedict's qualitative sugar reagent.

#### DISCUSSION OF RESULTS.

We believe it customary to analyze blood sugar time curves from the standpoint of the fasting level, initial rise, maximal concentration, and return to fasting level.

*Fasting Level.*—Two-thirds of the experiments indicate a fasting level in the limits of 0.08 to 0.1 per cent; about one-third show levels between 0.1 and 0.12 per cent. In two experiments, values of 0.136 per cent (No. 10) and 0.155 per cent (No. 21) were found. The mean of twenty-five observations is 0.104.

*Initial Rise.*—An average of about eighteen samples was taken from one subject. In order not to cause too much discomfort, we refrained from a close study of the initial rise in the present work. The problem is, however, being investigated at present and will be ready for publication in the near future (6, 11, 19).

*Maximal Concentration.*—The shortest time interval in which the maximum of hyperglycemia in these experiments was reached is 15 minutes; the longest period, 49 minutes. The average of twenty-two results indicates that the maximal concentration was attained in 33 minutes. This mean agrees with the findings of the majority of observers (7, 9, 10, 11, 14, 15, 16, 17, 21); our shortest time is, however, somewhat longer than the intervals found by Hamman and Hirschman (8, 9), and our longest period is exceeded by the findings of Goto and Kuno (5).

Expressed in per cent, we find the lowest maximal concentration in the present work to be 0.136, the highest 0.206 per cent with a mean of 0.166 per cent. This average is somewhat higher

than the maxima found by Hopkins (10), Goto and Kuno (5), Gray (7), and others (9, 19, 20, 21).

In all the points discussed so far there is a fair agreement with the reports presented in the literature. There is, however, a distinct difference when we analyze the configuration of the curves obtained in the present investigation. If we accept the composite graph of Janney and Isaacson (12, 13) which summarizes their findings on seventeen normal individuals as comparatively typical, we observe a steep rise from 0.105 per cent to 0.135 per cent within 45 minutes, with a gradual and smooth drop back to the fasting level in about 2 hours. In the present results one notices the same steep ascent throughout, but in sixteen of the twenty-two experiments a fairly *rapid drop is followed by a second rise*; in four cases we even see a third peak. The second gradient approaches the maximum ascent in one-half of the cases. The third peaks are distinctly lower.

*Return to Fasting Level.*—All investigators seem to agree that the return to the normal level occurs within about 2 hours. In more than half of the present findings, the fasting level is not reestablished before  $2\frac{1}{2}$  hours. Moreover, it must be noted that in many of the graphs which show a secondary rise the fasting level is closely approached and that there is even a descent below this line (hypoglycemia) before the second ascent begins. Some of the curves show a rising tendency on reaching the preprandial niveau after  $2\frac{1}{2}$  hours or more.

*Blood Sugar of Fasting Individuals.*—The curves of the fasting subjects show very little fluctuation after the first or second sample was taken. If we discount the results of the first 15 minutes, we find that the variations keep within 0.01 per cent of the blood sugar. The slightly higher starts are evidently due to some excitement of the individual, because of the fact that the subsequent findings show a rather smooth line.

#### CONCLUSIONS.

In many of the carbohydrate tolerance curves presented in the literature we find that the samples were taken in half hour and 1 hour intervals. If we draw a line through the half hour, 1 hour, 2 hour, and 3 hour points of the present graphs showing second-



dary peaks, we obtain curves possessing a distinct second rise in only four out of sixteen cases. This may be acceptable as an explanation why the secondary rise has not been generally observed.

If we consider the present findings in the light of the theories of MacLean and de Wesselow (18), and of Foster (2, 3), we are led to assume that the first drop is due to the stimulation of the metabolic function, which function according to the above mentioned authors is chiefly the glycogenic activity of the liver. In view of the secondary rise, however, we must assume an overstimulation, possibly due to the large amount of sugar, the extent of this overstimulation being so great as to cause something in the nature of a fatigue, thereby allowing the sugar in the blood to rise *again*, before the functional activity has been fully reestablished.

Folin and Berglund (1) advance the theory that the absorption by the tissues rather than the glycogen formation plays the chief rôle in the regulation of the blood sugar level after the ingestion of sugar. In the present work there is a return to nearly the fasting level, or even hypoglycemia (without glycosuria). If this indicates a decreased need for sugar transportation from tissue to tissue, then the secondary rise would not be apt to occur unless we assume selectivity of tissues.

#### SUMMARY.

Postprandial hyperglycemia is studied on normal individuals. Fifteen to twenty-two blood samples are taken within a 3 hour period. The blood sugar time curves show wide fluctuations, and in many cases a secondary rise before a definite return to the fasting level.

In conclusion we wish to thank the students, who volunteered as donors in this work, for their kindness.

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## Blood Sugar Studies

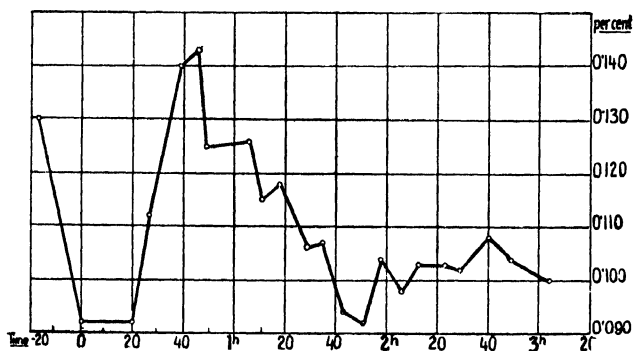


CHART 1. M. H. B., age 24 years, weight 170 pounds. 130 gm. glucose in 400 ml. H<sub>2</sub>O given at 33°C. 2 gm. citric acid as flavoring. Urine negative.

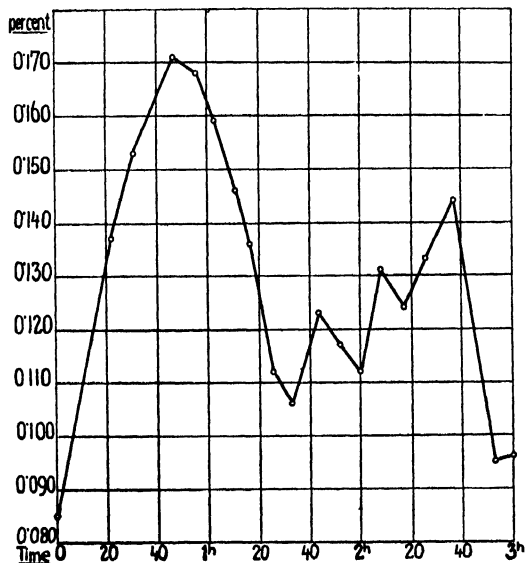


CHART 2. S. L. C., age 22 years, weight 140 pounds. 111 gm. glucose in 350 ml. H<sub>2</sub>O given at 37.5°C. Urine negative.

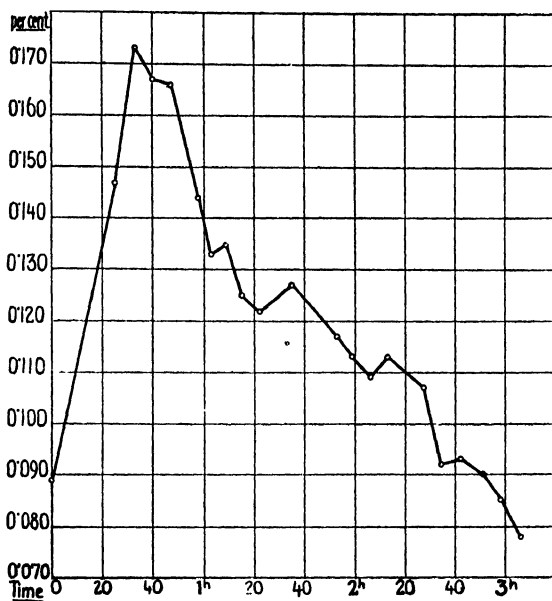


CHART 3. L. S. C., age 23 years, weight 130 pounds. 105 gm. glucose in 400 ml.  $H_2O$  given at  $37^\circ C$ . Urine negative.

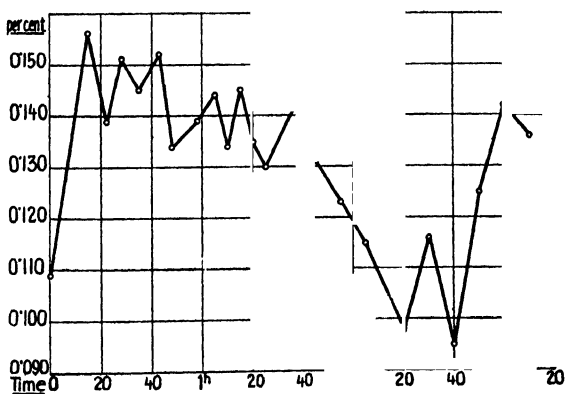


CHART 4. E. F. D., age 23 years, weight 160 pounds. 128 gm. glucose in 400 ml.  $H_2O$  given at  $37.5^\circ C$ . Urine negative.

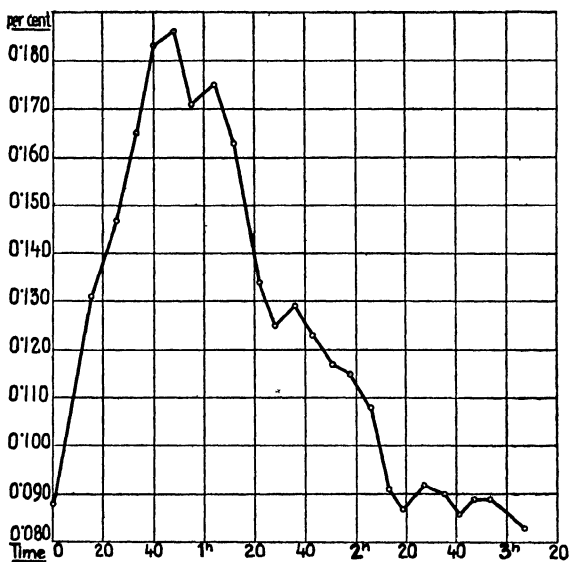


CHART 5. J. E. H., age 24 years, weight 140 pounds. 111 gm. glucose in 350 ml.  $H_2O$  given at  $37^\circ C$ . Urine negative.

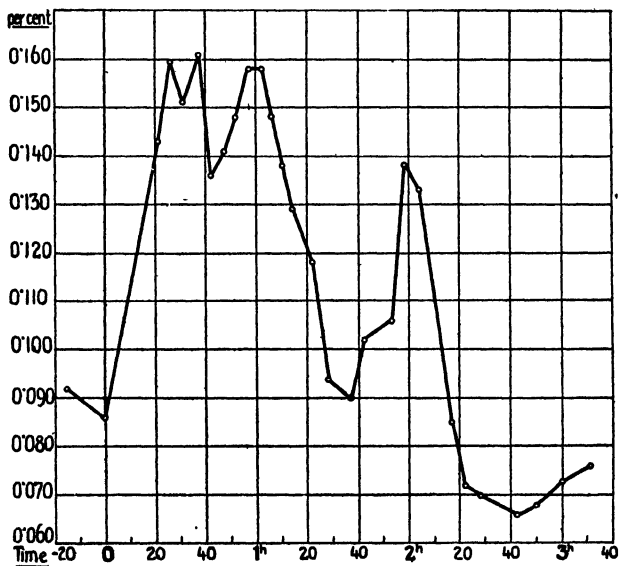


CHART 6. M. G., age 34 years, weight 165 pounds. 131 gm. glucose in 400 ml.  $H_2O$  given at  $37^\circ C$ . Urine negative.

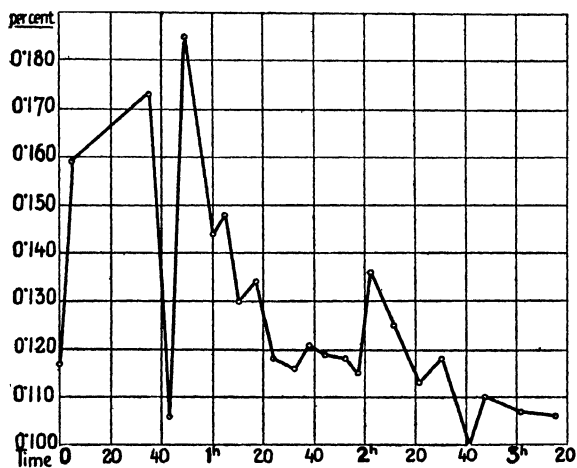


CHART 7. N. B. K., age 22 years, weight 155 pounds. 123 gm. glucose in 400 ml.  $H_2O$  given at  $37^{\circ}C$ . Nephritic; urine: albumin, casts, no sugar.

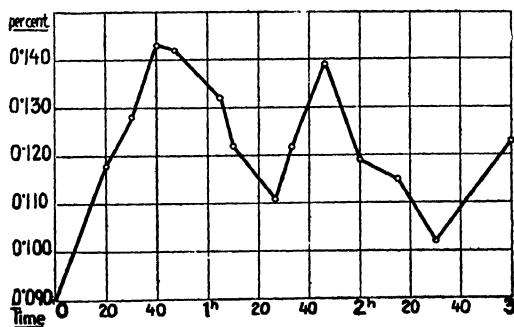


CHART 8. F. C. K., age 20 years, weight 140 pounds. 112 gm. glucose in 400 ml.  $H_2O$ , iced. Urine negative.

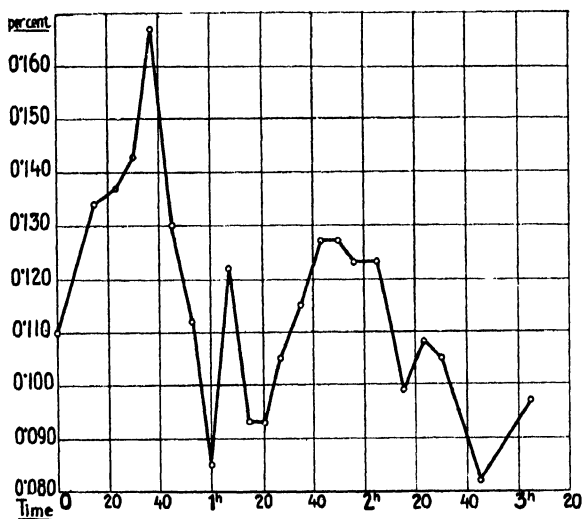
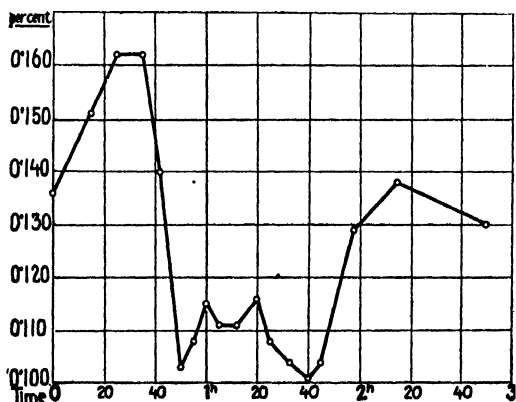


CHART 9. A. E. K., age 23 years, weight 160 pounds. 127 gm. glucose in 400 ml.  $H_2O$  at  $45^\circ C$ . Urine negative.



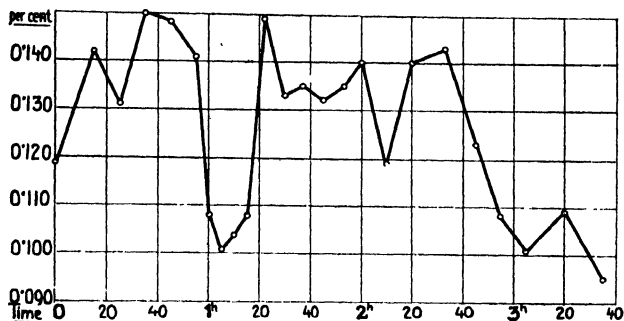


CHART 11. A. L., age 22 years, weight 130 pounds. 105 gm. glucose in 400 ml.  $H_2O$ , iced. Urine negative.

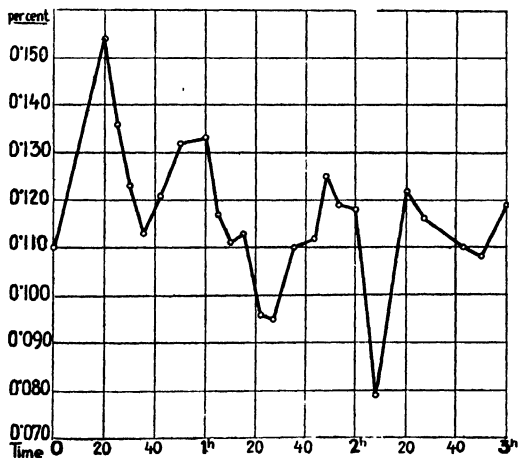


CHART 12. A. L., age 22 years, weight 130 pounds. 105 gm. glucose in 400 ml.  $H_2O$  given at  $37.5^{\circ}C$ . Urine negative.



## Blood Sugar Studies

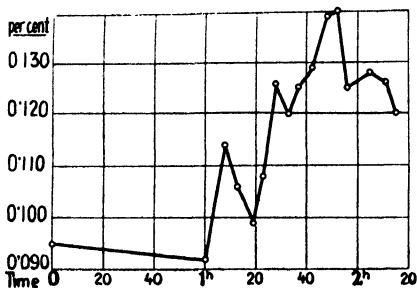
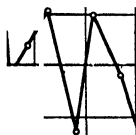


CHART 13. P. J. M., age 25 years, weight 150 pounds. 123 gm. glucose in 400 ml.  $H_2O$  given at  $37^\circ C$ . Urine negative.



0.100

20 40 1<sup>h</sup> 20 40 2<sup>h</sup> 20 40 3<sup>h</sup> 20

CHART 14. C. J. M., age 23 years, weight 162 pounds. 129 gm. glucose in 400 ml.  $H_2O$  given at  $37^\circ C$ . Urine negative.

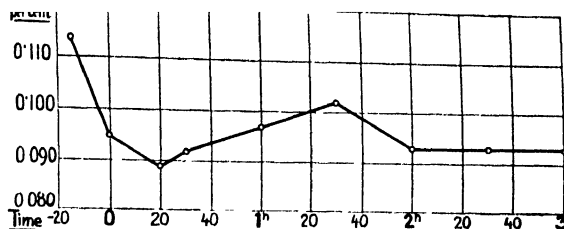


CHART 15. A. H. O., age 21 years, weight 138 pounds. Stomach empty, nothing given. Urine negative.

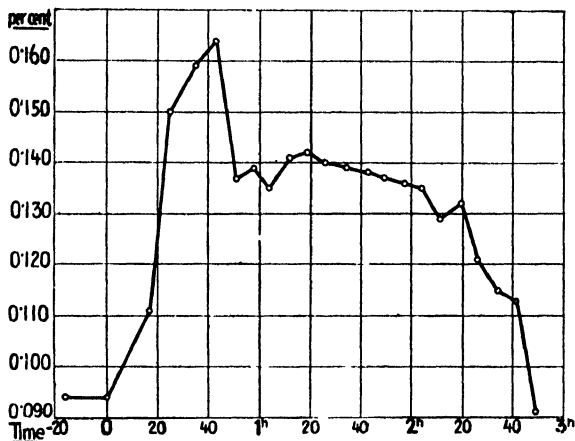


CHART 16. E. A. R., age 22 years, weight 160 pounds. 122 gm. glucose in 350 ml.  $H_2O$  given at  $37^{\circ}C$ . Urine negative.

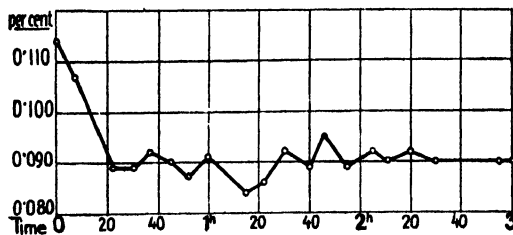


CHART 17. E. A. R., age 22 years, weight 160 pounds. Empty stomach, nothing taken. Urine negative.

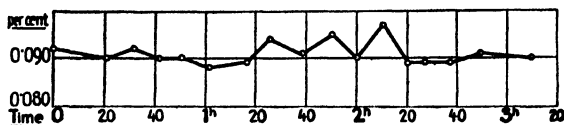


CHART 18. E. J. S., age 23 years, weight 130 pounds. Drank 400 ml.  $H_2O$  at  $15^{\circ}C$ . Urine negative.

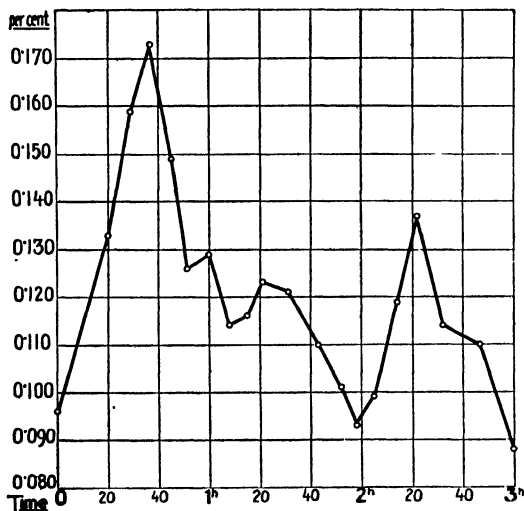


CHART 19. E. J. S., age 23 years, weight 130 pounds. 125 gm. glucose in 440 ml.  $H_2O$  plus 1 gm. citric acid, as flavoring, given at  $37^{\circ}C$ . Urine negative.

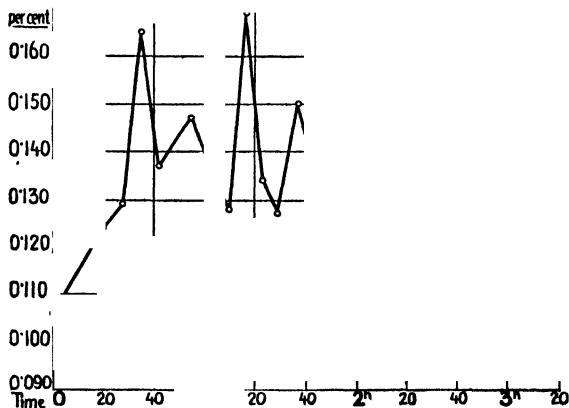


CHART 20. E. d.C. S., age 22 years, weight 120 pounds. 120 gm. glucose in 400 ml. ice water. Urine negative.

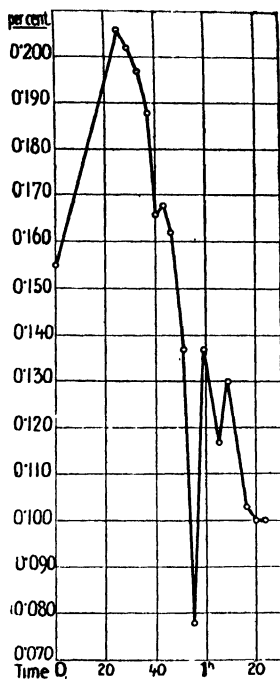


CHART 21. G. R. S., age 20 years, weight 157 pounds. 120 gm. glucose in 400 ml.  $H_2O$  given at  $37.5^{\circ}C$ . Urine negative.

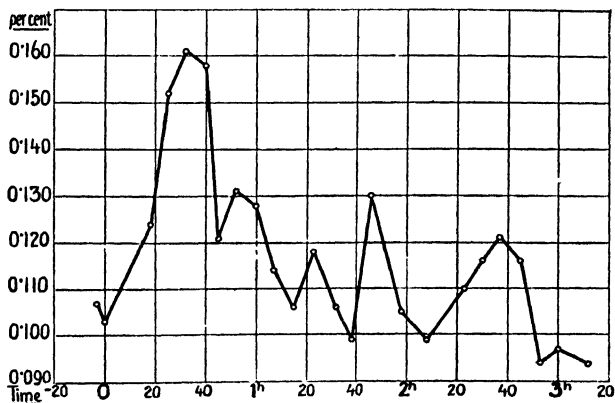


CHART 22. G. R. S., age 20 years, weight 160 pounds. 120 gm. glucose in 400 ml.  $H_2O$  given at  $37.5^{\circ}C$ . Urine negative.

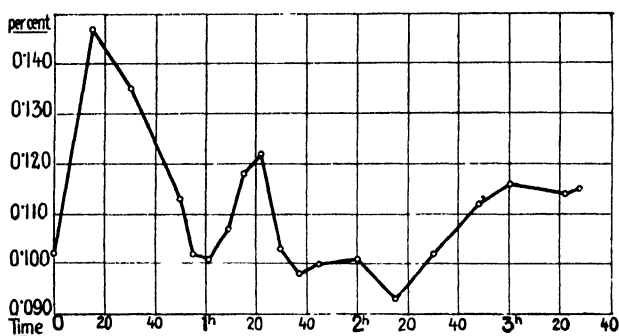


CHART 23. J. V. W., age 23 years, weight 135 pounds. 110 gm. glucose in 400 ml. ice water. Urine negative.

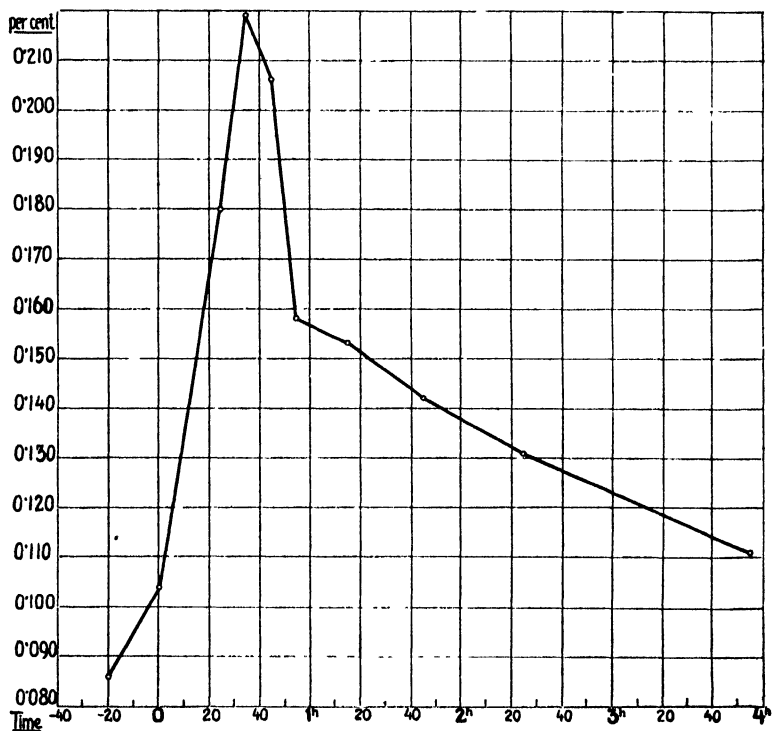


CHART 24. L. H. G., age 24 years, weight 138 pounds. 110 gm. glucose in 400 ml. H<sub>2</sub>O given at 17°C. Juice of two lemons as flavoring. Urine negative.

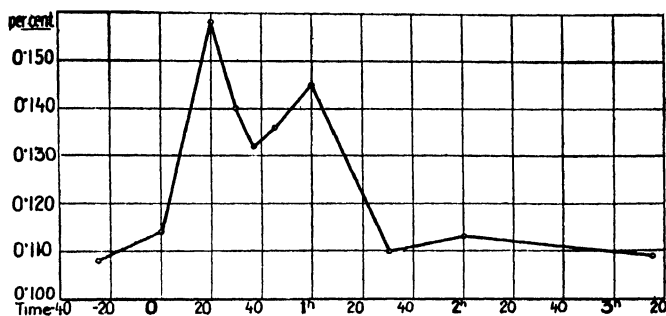


CHART 25. A. E. N., age 20 years, weight 160 pounds. 127 gm. glucose in 400 ml.  $H_2O$  given at  $0^\circ C$ . Juice of two lemons as flavoring. Urine negative.

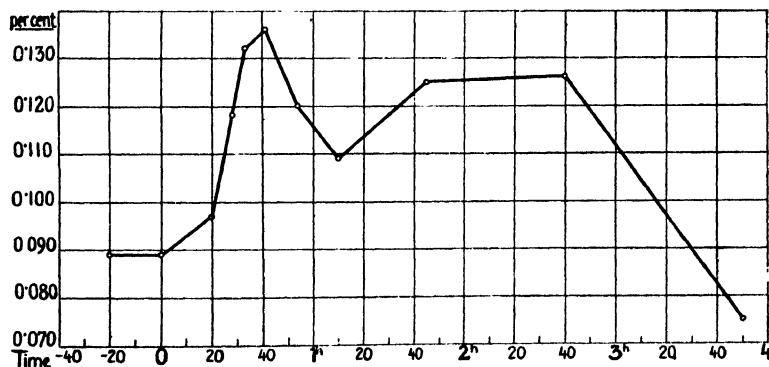


CHART 26. H. G. W., age 24 years, weight 145 pounds. 115 gm. glucose in 400 ml.  $H_2O$  given at  $0^\circ C$ . Juice of two lemons as flavoring. Urine negative.



# THE INFLUENCE OF HYDROGEN ION CONCENTRATION AND OF TEMPERATURE ON THE HYDROLYTIC SCISSION OF CASEIN.

By D. C. CARPENTER.

*(From the Chemical Laboratory of the New York Agricultural Experiment  
Station, Geneva.)*

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In the course of an investigation to perfect a method for obtaining pure casein from milk, it became apparent that, in order to produce a pure protein which had not suffered hydrolytic scission during its process of preparation, a knowledge of the effects of acid and alkali over a considerable range of concentration and also the influence of temperature was essential. The removal from crude casein of certain impurities which are comparatively insoluble in water does not appear to be possible without resorting to resolution of the casein in some solvent, either acid or alkali, in which at least part of the impurities are insoluble and in which form they are capable of mechanical elimination. It was primarily to establish working conditions so as to preclude as nearly as possible any chance of hydrolytic cleavage of casein during purification that the present investigation was undertaken. Numerous investigators have reported the products of complete hydrolytic cleavage of casein, but any knowledge of the conditions under which casein is not hydrolyzed appears to be wanting.

That complete hydrolysis of casein is not effected until boiled with strong hydrochloric acid for more than 24 hours has been shown by Osborne and Guest (1). These authors found that appreciable amounts are still undigested even after 96 hours of vigorous boiling in strong hydrochloric acid. Plimmer and Bayliss (2), experimenting on the splitting off of soluble  $P_2O_5$  from casein by the action of alkali, found that very dilute alkali, sufficient only to dissolve the casein, caused no splitting off of soluble  $P_2O_5$ . However, 15 per cent HCl and also 1 per cent solu-



tions of caustic soda were found to cause splitting. These workers found, however, that the latter reagent scarcely increased the soluble nitrogen and concluded that the effect of 1 per cent alkali was confined chiefly to the splitting off of  $P_2O_5$  from casein.

It has been stated by Robertson (3) that "we must conclude that neutral calcium caseinate in watery solution undergoes fairly rapid hydrolysis, *one-third of the substrate being hydrolyzed in twelve hours. I have also observed that neutral sodium caseinate undergoes a similar hydrolysis in watery solution*, but I have not determined the actual value of the velocity-constant." (The italics are our own.) If the foregoing statement is founded on fact, then it would be useless to attempt to purify casein by solution in alkali followed by reprecipitation.

A more extensive study of the autohydrolysis of caseinates has been made by Walters (4) in which the ratios of casein to base were  $1:50 \times 10^{-5}$  and  $1:80 \times 10^{-5}$  equivalents, the former being a neutral and the latter a basic caseinate. Walters concluded that the neutral caseinates of lithium, sodium, and potassium in sterile solution undergo comparatively rapid autohydrolysis, about 5 per cent being hydrolyzed in 96 hours at  $37.5^\circ\text{C}$ . Basic caseinates of the above alkali metals behaved similarly with slightly higher velocities. The basic caseinates of barium and calcium were found to hydrolyze about three times as rapidly as the corresponding alkali metal salts. In the experiments reported Walters obtained his figure for the unhydrolyzed casein present after a given time by reprecipitating the unchanged casein at the isoelectric point by adding dilute acetic acid. This procedure, in the opinion of the writer, is objectionable for the reason that complete reprecipitation of the casein is impossible unless the hydrogen ion concentration of the solution be accurately controlled. This rigid control is impossible without the use of electrometric measurements.

The method whereby unhydrolyzed protein is precipitated by 2.5 per cent  $\text{CCl}_3\text{COOH}$ , used by Hiller and Van Slyke (5) and since adapted by other workers into a general scheme for separating hydrolysis products from the parent protein, is much to be preferred in the case of casein to any method depending on separating the parent protein in an isoelectric condition from its hydrolysis products. Cohn (6) reports that uncombined casein is

soluble in water to the extent of 17.8 mg. of nitrogen per liter. The writer has found the solubility of casein in 2.5 per cent  $\text{CCl}_3\text{COOH}$  to be equal to 2.43 mg. of nitrogen per liter. The choice is entirely in favor of  $\text{CCl}_3\text{COOH}$  so far as completeness of removal of casein nitrogen from solution is concerned.

The problem can be gotten at in another way; namely, by Sørensen's formol titration method, in which it is not necessary to separate the hydrolyzed from the unhydrolyzed portion, and it seemed advisable to use this method in the present investigation in connection with the  $\text{CCl}_3\text{COOH}$  method. In Sørensen's formol titration method the amino groups are converted into methylene amino groups by the action of formaldehyde, and the resulting increase in free carboxyl groups is titrated with alkali, one equivalent weight of alkali being equivalent to one amino group. This method is more applicable in the case of casein than the Van Slyke method for amino nitrogen on account of the unwieldy coagulum obtained in the latter method.

Both of the methods employed in this work are fundamentally based on reactions involving nitrogen. It is to be pointed out that the casein molecule also contains sulfur and phosphorus both of which are liable to suffer scission from hydrolysis, and hence any measure of scission of the molecule based on any one elementary constituent may not enable us to point out conditions bearing on the scission of other constituents.

#### EXPERIMENTAL.

##### *Trichloroacetic Acid Method.*

In preparing the casein solutions used on the acid side of the isoelectric point, a weighed sample of casein (prepared by a method to be described in a forthcoming publication), containing as its only inorganic impurity 0.012 per cent  $\text{SiO}_2$ , was shaken several hours with cold water until thoroughly wetted. Then 5.00 cc. of  $\text{N}/10$   $\text{HCl}$  per gm. of casein were added, in small portions at a time, with vigorous shaking after each addition. 80 cc. samples of this stock solution, containing about 1.25 per cent casein at approximately pH 3.2, were pipetted into 100 cc. volumetric flasks, and further acid was added, dropwise with shaking, to change the pH to lower values which would cover the acid range

at intervals up to approximately pH 1, and each flask was then filled to the mark with water so that all the experiments would be conducted on solutions containing 1.00 per cent casein. At pH values slightly less than 1 casein is precipitated, presumably as a hydrochloride, and hence we are prevented from going into the region of higher acid concentration. In the experiments reported below the casein is entirely in solution at all pH values, the experiments at pH 1.04 being very near the pH limit at which precipitation is encountered.

The stock casein solution for use on the alkaline side of the isoelectric point was made from a weighed amount of the same sample of casein as above, wetted first with cold water, and then dissolved by adding 4.80 cc. of  $N/10$  NaOH (carbonate-free) per gm. of casein in small lots at a time, shaking vigorously after each addition. This stock solution was made to contain 2 per cent casein and had a pH value in the vicinity of pH 6.5. 50 cc. samples of this solution were pipetted into 100 cc. volumetric flasks and varying amounts of standard NaOH solution were added dropwise to each to change the pH to higher values. Then each was diluted to 100 cc. so that the casein content of the hydrolyzing solution was 1.00 per cent, the same casein concentration used in experiments on the acid side of the isoelectric point. The solution for pH 5.86 was prepared by cautious additions of  $N/10$  HCl to the stock solution with violent agitation. This represents the limit possible without precipitation of casein from solution. This solution contains, as a result of the neutralization mentioned, NaCl to the extent of 0.0024  $N$ . Such a small concentration as this can hardly have any catalytic influence on the reaction.

Both of the above mentioned stock solutions were analyzed in triplicate for nitrogen by the Kjeldahl-Gunning method as a check on the actual casein content of the solutions.

One of the factors to be guarded against in any work with protein solutions is bacterial contamination. The writer avoided this by using sterilized glassware and recently boiled water for preparing solutions and protected the solutions with a few drops of toluene in tightly stoppered flasks during the course of experiments. All solutions herein reported were free from bacterial contamination at the end of the 6 day hydrolysis period as determined by direct microscopic observation.

The initial pH of the series of solutions was determined potentiometrically within an hour after the solutions were prepared, using fresh hydrogen electrodes in the Clark cell and freshly prepared tenth normal calomel reference electrodes. Junction was made with saturated potassium chloride solution. The potentiometer used was the Leeds and Northrup type K instrument and the null instrument, a highly sensitive type R galvanometer from the same manufacturer. The standard cell was calibrated at the Bureau of Standards. Each hydrogen electrode before use with protein solutions was checked in  $m/20$  potassium acid phthalate solution against the tenth normal calomel electrodes. In case an electrode failed to give the standard potential (Clark (7)), against the phthalate solution, it was discarded.

The series of flasks containing the casein solutions at various hydrogen ion concentrations were placed at once in constant temperature baths at  $5^{\circ} \pm 1.0^{\circ}\text{C.}$ ,  $25^{\circ} \pm 0.002^{\circ}\text{C.}$ ,  $40^{\circ} \pm 0.02^{\circ}\text{C.}$ , and  $60^{\circ} \pm 0.50^{\circ}\text{C.}$ , respectively, and allowed to remain for 3 and 6 days for hydrolytic action to take place. At the end of this time a 20 cc. sample was removed from each flask, transferred to a 50 cc. volumetric flask, treated with 12.50 cc. of a 10 per cent solution of  $\text{CCl}_3\text{COOH}$  to precipitate the unhydrolyzed casein. The volume was then made up to the 50 cc. mark with water. The flask was thoroughly shaken and allowed to stand 15 minutes for the precipitation reaction to become complete. Hiller and Van Slyke (5) have shown that 15 minutes contact of this concentration of  $\text{CCl}_3\text{COOH}$  has very little hydrolytic action on proteins. The contents of the flask was then filtered through paper, the filtrate containing practically all of the hydrolyzed portion of the protein. No attempt was made to wash the paper or unhydrolyzed casein precipitate with water after filtration, and the results reported for "nitrogen hydrolyzed" are presumably a little low on this account. A greater error would have been made, however, if washing with water had been attempted and unhydrolyzed casein thereby dissolved and added to the filtrate. The filtrate was then analyzed for nitrogen by the usual Kjeldahl-Gunning method. The filtrates in all cases were water-clear and showed no opalescence, evidence of freedom from unhydrolyzed casein.

*Formol Titration Method.*

The solutions used in following the course of the hydrolysis by the formol titration method were made in the same manner as those just described except that slightly larger quantities of standard HCl and NaOH solutions were used in preparing the stock solutions. This simply widens the interval between the curves for the acid and alkaline hydrolysis.

Experiments were conducted only at 25, 40, and 60°C. in this series, and the hydrolysis was determined after 6, 12, and 18 days. In carrying this method out for the formol titration values, 20 cc. samples were taken from each flask and a drop of phenolphthalein indicator added to each. These samples were brought to a faint pink color by the addition of acid or base as was required, formolized with 10 cc. of 40 per cent formaldehyde solution (likewise neutralized to phenolphthalein) and after 15 minutes titrated again to a faint pink color with  $N/10$  KOH.

The opalescence of the casein solutions renders the end-point indistinct except in good light, and the straw-color of the solutions which are strongly hydrolyzed materially detracts from the usefulness of this method. Duplicability of results is poor by this method and much inferior to the trichloroacetic acid method.

The recorded titration value includes the formol titration of both hydrolyzed and unhydrolyzed casein present. The formol titration of 1 gm. of casein alone which has undergone practically no hydrolysis was found to be 4.65 cc. of  $N/10$  KOH. This value may be considered as a blank in the above titrations.

It also has a definite bearing on the weight of the casein molecule. This tells us that by the formolization reaction 0.000465 of a formula weight of an amino group per gm. of casein has been converted into methylene amino group. 2150 gm. of casein would be required to contain one amino group thus reacting (one COOH group thereby being freed from inner salt formation). The molecular weight of casein would appear to be some multiple of 2150. Cohn and Hendry (8) have found the combining weight of casein for bases by solubility methods to be about 2100. And Greenberg and Schmidt (9) have found from transference data 2015 gm. of casein to be associated with unit electrical charge in solutions of alkali saturated with casein.

TABLE I.  
*Casein Hydrolyzed in 3 Days.*

Solution.	Initial pH.	Nitrogen hydrolyzed per 1 gm. casein.			
		5°C.	25°C.	40°C.	60°C.
		<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	1.04	0.00	0.56	4.80	47.20
2	1.43		0.31	2.65	25.70
3	2.00	0.00	0.27	1.94	14.70
4	2.60		0.22	1.74	8.80
5	3.05	0.00	0.22	1.58	6.92
6	5.86	0.00	0.32	1.60	2.55
7	6.76		0.68	2.80	3.90
8	7.53	0.00	0.97	3.79	5.58
9	8.40		1.20	4.52	6.41
10	9.25	0.00	1.27	4.80	7.05
11	10.01		1.38	5.10	8.50
12	10.65	0.19	1.75	6.52	13.40
13	11.15	0.76	3.82	10.31	23.10
14	11.74	1.12	5.12	11.93	27.60

TABLE II  
*Casein Hydrolyzed in 6 Days.*

Solution.	Initial pH.	Nitrogen hydrolyzed per 1 gm. casein.			
		5° C.	25°C.	40°C	60°C.
		<i>mg.</i>	<i>mg</i>	<i>m /</i>	<i>mg.</i>
1	1.04	0.00	1.95	8.30	75.50
2	1.43		1.00	5.00	45.95
3	2.00	0.00	0.81	3.14	28.90
4	2.60		0.72	2.41	14.98
5	3.05	0.00	0.70	2.18	11.90
6	5.86	0.00	0.65	3.12	4.87
7	6.76		1.20	5.10	6.20
8	7.53	0.00	1.94	6.12	8.62
9	8.40		2.24	6.76	10.10
10	9.25	0.00	2.45	7.18	11.35
11	10.01		2.74	7.80	14.50
12	10.65	0.40	3.32	10.45	20.14
13	11.15	1.20	6.12	14.52	31.30
14	11.74	1.83	7.38	17.68	39.75

*Results.*

The measure of the hydrolytic scission, so far as nitrogen is concerned, which has taken place in 3 and 6 days, respectively,

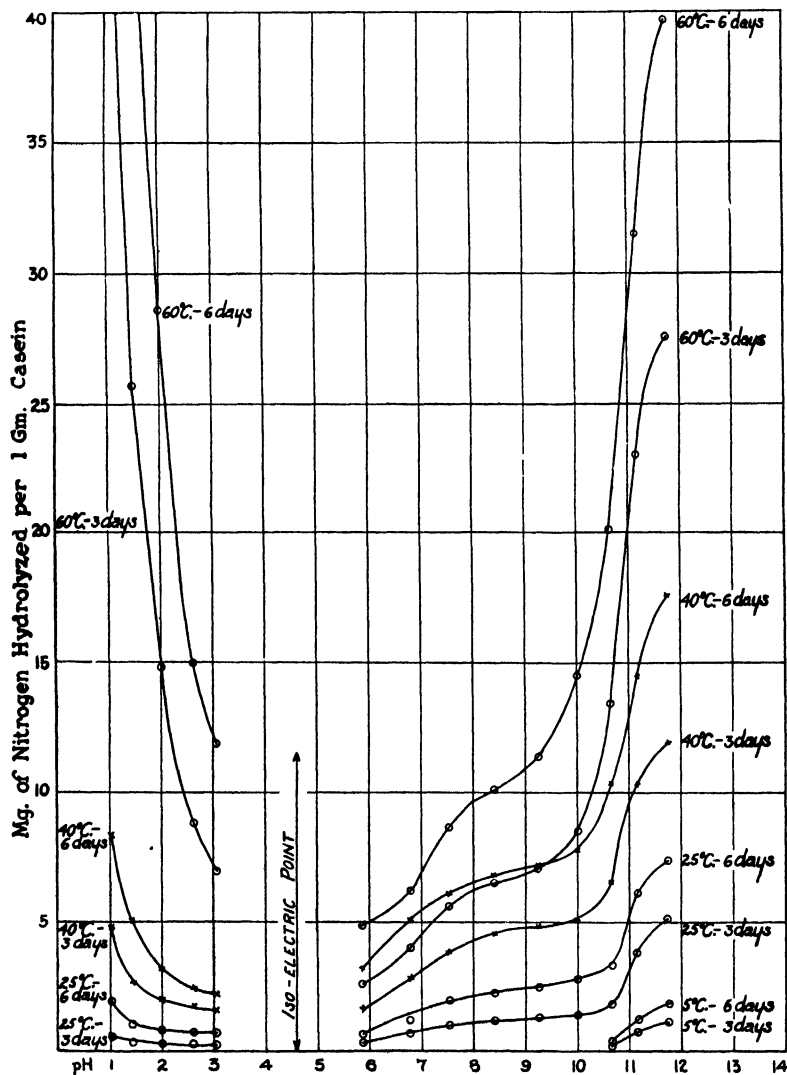


FIG. 1.

determined by the  $\text{CCl}_3\text{COOH}$  method, is recorded in Tables I and II and shown graphically in Fig. 1. The hydrolysis as measured by the formol titration method, is recorded in Table III and the hydrolysis after 12 days is graphically expressed in Fig. 2. It is impracticable to prepare solutions of casein in the range pH about 3.5 to 5.8 due to its insolubility, this interval being the approximate isoelectric zone for this protein.

TABLE III.  
*Formol Titration Values.*

Solution.	Initial pH.	N/10 KOH required per 1 gm. casein.								
		25°C.			40°C.			60°C.		
		6 days.	12 days.	18 days.	6 days.	12 days.	18 days.	6 days.	12 days.	18 days.
		cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
1	0.95	5.10	5.62		6.85	8.62		9.22	10.52	
2	1.38	5.13	5.35		5.93	7.07		7.77	8.70	
3	2.55	4.90	5.06		5.44	5.88		5.80	6.43	
4	6.10	4.92	5.20	5.62	4.93	5.42	6.13	4.95	5.62	6.29
5	7.16	5.37	5.54	5.72	5.24	5.56	6.19	5.40	6.00	6.60
6	8.30	5.60	5.71	5.83	5.72	5.98	6.24	6.01	6.41	6.81
7	9.15	5.77	5.78	5.78	5.92	6.05	6.19	6.32	6.54	6.76
8	10.15	5.77	5.88	5.98	6.13	6.44	6.75	6.64	6.73	6.81
9	11.15	5.87	6.26	6.65	6.70	6.99	7.27	6.90	7.11	7.48
10	11.85	5.87	6.41	6.96	7.06	7.37	7.68	7.31	7.85	8.40
11	12.38	6.29	6.78	7.27	7.30	7.65	8.00	7.77	8.42	9.07

The data accumulated by the  $\text{CCl}_3\text{COOH}$  method indicate in a much more conclusive manner than those by the formol titration method the effects of temperature and hydrogen ion concentration on the hydrolysis of casein. In general, the two methods agree with each other in showing the hydrolysis which has taken place in the various solutions, but the former method is much more clear-cut; discussion, therefore, will be chiefly confined to the results obtained by that method. Duplicates of nitrogen hydrolyzed by the  $\text{CCl}_3\text{COOH}$  method agreed with each other within 0.02 cc. of N/10  $\text{H}_2\text{SO}_4$  in the titration, except in four cases where the difference was less than 0.05 cc. of N/10  $\text{H}_2\text{SO}_4$ .



It is to be pointed out that as only one casein concentration was used throughout, we cannot say definitely to what order of reaction the hydrolysis of casein belongs. The agreement between the velocity constants for different times indicates, however, that the reaction on the acid side of the isoelectric point is of the first order. From the curves it is obvious that on the acid side of the isoelectric point the velocity constant is a regular function of the hydrogen ion concentration, except at low temperatures where no hydrolysis occurs, or so little as to be unmeasurable by the trichloroacetic acid method.

On the alkaline side of the isoelectric point the curves show the hydrolysis to be a more complicated function of the hydrogen

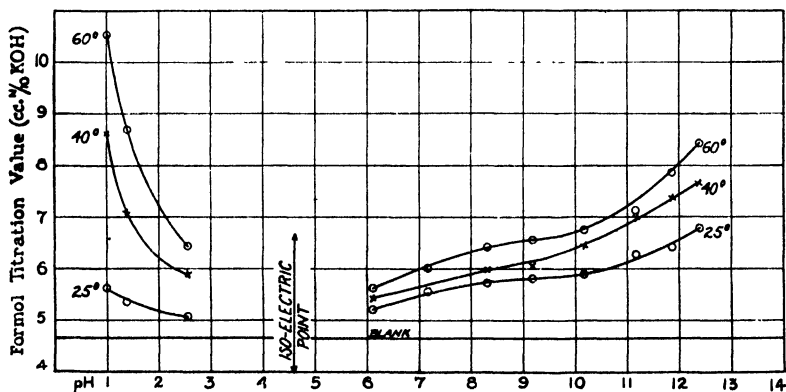


FIG. 2.

ion concentration, and with the meager data at our disposal a mathematical treatment is impossible. It is suggested that on the alkaline side we are dealing with the hydrolysis of two different types of compounds of casein, one the ordinary type of salt where base (sodium in the present case) replaces hydrogen in a COOH group, which replacement is usually complete for weak acids at about pH 9.0; and another type of salt where base is possibly combined at the CONH linkages through a keto-enol tautomeric equilibrium, this latter type of salt occurring at pH values above 9.0 and being presumably more readily hydrolyzable than the former type.

In passing, it is of interest to note that the point of minimum

hydrolytic scission of casein is in the vicinity of pH 5.0. This fact supports the hypothesis advanced by Rice (10) that hydrolysis should be at a minimum in this region rather than at pH 7.0, due to the unequal hydration of the hydrogen and hydroxyl ions.

Zoller (11) in studying the viscosity of casein solutions, attributed the decrease in viscosity of solutions more alkaline than pH 9 to hydrolysis of the casein. He failed to obtain a decrease in viscosity in solutions where  $\text{NH}_4\text{OH}$  was used as solvent but obtained the decrease where the alkali metal hydroxides were used. The solutions for his work were held at  $30^\circ\text{C}$ . for 2 hours during solution of the casein in alkali. How much longer they were held or at what temperature before the viscosity was measured is not stated. From our own data it would appear that a 1 per cent casein solution at pH 11.74 and  $40^\circ\text{C}$ . in 3 days hydrolyzed only to the extent of 7.6 per cent, while at pH 9.0 for the same time and temperature the hydrolysis was 3 per cent. In the writer's opinion this relatively small increase in hydrolysis between the two pH values can in no way explain the recorded 50 per cent drop in viscosity within, presumably, a much shorter time interval and at a considerably lower temperature. It is believed that the viscosity changes mentioned will eventually find an explanation based on the type of protein compound predominant at a given pH, together with its water-adsorbing properties, rather than in the small amount of hydrolytic scission the protein molecule itself may have suffered.

#### SUMMARY.

The hydrolytic scission of a very pure casein has been measured in terms of nitrogen hydrolyzed over the pH ranges 1 to 3 and 6 to 12 for temperatures of 5, 25, 40, and  $60^\circ\text{C}$ . for hydrolysis periods of 3 and 6 days. These experiments were conducted on solutions of 1 per cent casein content and are shown in graphs and tables. Hydrolysis at temperature above  $5^\circ\text{C}$ . on the acid side of the isoelectric point of casein appears to follow a reaction of the first order at a given pH value and bears a simple relationship to pH.

Hydrolysis on the alkaline side of the isoelectric point bears a complex relationship to pH which was not ascertained mathematically. The shape of the hydrolysis curve suggests that two

types of protein compounds are present, one existing above pH 9 and easily hydrolyzed and the other existing below pH 9 and less readily hydrolyzed.

At 5°C. no measurable hydrolysis of casein occurs in 6 days except at pH values greater than 10. Casein may be handled in 1 per cent solutions without undergoing hydrolytic scission in acid solutions between pH 1 and 3 or in solutions of alkalis between pH 6 and 10, provided the temperature be kept in the vicinity of 5°C. At 25°C. 1 per cent solutions of casein in alkali at pH 7.0 hydrolyze about 0.15 per cent per day and in acid at pH 3.0 hydrolyze about 0.08 per cent per day.

Obviously, the pasteurization of milk or the warming of milk or casein solutions from which unhydrolyzed casein is to be later prepared is to be avoided.

From formol titration data it is shown that one formula weight of amino group is linked with carboxyl per 2150 gm. of casein. In other words, the molecular weight of casein is some multiple of this number.

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## BROMOLECITHINS. II.

### BROMOLECITHINS OF THE LIVER AND EGG YOLK.

By P. A. LEVENE AND IDA P. ROLF.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

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From the work of Levene and Ingvaldsen,<sup>1</sup> Levene and Simms,<sup>2,3</sup> and Levene and Rolf,<sup>4, 5, 6, 7, 8</sup> the following new facts regarding lecithin chemistry came to light. First, the amino nitrogen-free fraction of the unsaturated phosphatides on hydrolysis yielded two saturated fatty acids, namely stearic and palmitic, and several unsaturated fatty acids. Among the latter arachidonic and oleic acid from the liver lecithin<sup>3</sup> were definitely identified. From the same lecithin linolenic acid was prepared in nearly pure state as the hexabromide. The presence of linolic acid was less certain. In the material obtained on hydrolysis of egg lecithin, three unsaturated fatty acids were identified, namely arachidonic, linolic, and oleic.<sup>5, 6</sup> Whether the lecithin fraction of the liver and that of the egg yolk contain all the four unsaturated fatty acids cannot be stated with certainty at present. It is however certain that linolenic acid predominates in the lecithin fraction of the liver and linolic in that of the egg yolk. This conclusion is made more certain by the findings to be communicated in this paper.

There is, however, little doubt that every lecithin fraction

<sup>1</sup> Levene, P. A., and Ingvaldsen, T., *J. Biol. Chem.*, 1920, xliii, 359.

<sup>2</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1921, xlviii, 185.

<sup>3</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1922, li, 285.

<sup>4</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xli, 193.

<sup>5</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1921, xli, 353.

<sup>6</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 507.

<sup>7</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, liv, 91.

<sup>8</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1924-25, lxii, 759.

obtained from either plant or animal tissues contains two saturated and several unsaturated acids.

In a review of the literature on lecithin one of us (L.)<sup>9</sup> discussed fully the question as to whether our present knowledge of the structure of lecithins permits the concept of a very complex molecule containing the two saturated and the several unsaturated acids. In this place, let it suffice to state that the molecular weight for hydrogenated lecithin found by Levene and Simms<sup>10</sup> indicates that the molecule of lecithin is composed of one glycerophosphoric acid in combination with two fatty acids and with one base.

Yet it seemed desirable to obtain additional evidence in favor of the simple structure of lecithin; also to ascertain whether every stearyl lecithin exists in as many forms as the number of accompanying unsaturated acids and whether every palmityl lecithin exists in as many forms. The method of bromination was applied<sup>11</sup> after several unsuccessful attempts to fractionate further the lecithin fraction. By this method the following results were obtained. Two bromolecithin fractions were isolated from the liver, one of which analyzed as an octabromo derivative and the other as a hexabromo derivative. The derivatives with a lower bromine content were not isolated in a pure state. From the fraction which analyzed as an octabromolecithin two saturated fatty acids were obtained, namely palmitic and stearic. From the hexabromolecithin fraction the same two saturated fatty acids were prepared. A dibromolecithin and a tetrabromolecithin were isolated from the egg lecithin. The tetra derivative was hydrolyzed and was found to contain two saturated fatty acids.

Thus, as far as analyzed, each unsaturated fatty acid occurs in combination with each of the two saturated acids. This would signify that each unsaturated fatty acid is present in two lecithins. If the same should hold for the other unsaturated fatty acids, it would be necessary to assume the presence of eight lecithins in the liver and perhaps as many in other organs and in egg yolk.

The details of the method of separation of the bromolecithins

<sup>9</sup> Levene, P. A., *Physiol. Rev.*, 1921, i, 327.

<sup>10</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1921, xlviii, 196.

<sup>11</sup> Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1925, lxxv, 545.

will possibly have to be modified to suit each organ. The details thus far developed are given in the experimental part.

Mention, however, may be made of the method used by us for the separation of the saturated from the unsaturated fatty acids. This method may prove useful in other work as well. The soluble fraction of the brominated material containing the brominated acids of lower bromine content and the saturated fatty acids is esterified. Since the esters of saturated fatty acids boil at a lower temperature than those of the bromo acids, they are readily separated by fractionation. The fraction boiling below 180°C. at 1.0 mm. pressure contains the esters of the saturated acids.

#### EXPERIMENTAL.

*The Bromination of Liver Lecithin.*—Amino-free lecithin cadmium chloride was prepared from the acetone extract of 100 pounds of beef liver and decomposed with methyl alcoholic ammonia in the usual manner. The freed lipoid analyzed as follows:

No. 42. 0.1070 gm. substance: 0.2552 gm.  $\text{CO}_2$ , 0.0980 gm.  $\text{H}_2\text{O}$  and 0.0114 gm. ash.

0.2913 gm. substance: 0.0410 gm.  $\text{Mg}_2\text{P}_2\text{O}_7$ .

0.1942 " " required 2.95 cc. 0.1 N acid.

0.2194 " " (Wijs) absorbed 0.1682 gm.  $\text{I}_2$ .

Calculated. Oleyl stearyl lecithin,  $\text{C}_{44}\text{H}_{88}\text{O}_9\text{NP}$ .

C 65.55, H 11.01, N 1.73, P 3.85, iodine No. 31.

Found, No. 42. " 65.05 " 10.24 " 2.05 " 3.85 " " 76.

100 gm. of this lecithin were dissolved in a minimal quantity of ether, and the solution diluted with three volumes of gasoline. A solution of bromine in gasoline (40–60°) was added slowly, the temperature being maintained below  $-5^\circ$ . The brominated lipoid was precipitated by the addition of several volumes of gasoline.

This precipitate was fractionated by extraction with ether, 10 gm. of material remaining quite insoluble. The latter was a grayish powder as it separated from the ether, but coalesced in the desiccator to a dark brown, sticky mass which gave the analysis designated No. 51. The ethereal mother liquor was concentrated *in vacuo* to a small bulk. The addition of gasoline precipitated 15 gm. of material which was analyzed as No. 52.

- No. 51. 0.2014 gm. substance: 0.0168 gm.  $Mg_2P_2O_7$ .  
 0.1438 " " required (Kjeldahl) 1.30 cc. 0.1 N HCl.  
 0.1430 " " (Carius) 0.1638 gm. AgBr.
- No. 52. 0.2398 gm. substance: 0.0186 gm.  $Mg_2P_2O_7$ .  
 0.2056 " " required (Kjeldahl) 1.55 cc. 0.1 N HCl.  
 0.1486 " " (Carius) 0.1586 gm. AgBr.
- Calculated. Octabromosarachidyl stearic lecithin.  
 P 2.12, N 0.96, Br 43.72.
- Calculated. Octabromosarachidyl palmitic lecithin.  
 P 2.16, N 0.97, Br 44.57.
- Found, No. 51. " 2.32, " 1.16, " 48.75.  
 " " 52. " 2.16, " 1.04, " 45.42.

The mother liquor, after the separation of No. 52, was concentrated *in vacuo*, and from the residue, acetone precipitated light yellow flocks which analyzed as follows:

- No. 53. 0.3000 gm. substance: 0.0396 gm.  $Mg_2P_2O_7$ .  
 0.2000 " " required (Kjeldahl) 3.20 cc. 0.1 N HCl.  
 0.1182 " " (Carius) 0.0584 gm. AgBr.
- Found, No. 53. P 3.68, N 2.24, Br 21.02.

Concentration of the acetone mother liquor left a viscous residue. This, when poured into cold alcohol, deposited a dark lecithin-like precipitate which analyzed as follows:

- No. 54. 0.3000 gm. substance: 0.0256 gm.  $Mg_2P_2O_7$ .  
 0.2000 " " required (Kjeldahl) 1.60 cc. 0.1 N HCl.  
 0.1908 " " (Carius) 0.1584 gm. AgBr.
- Calculated. Hexabromostearyl stearic lecithin.  
 P 2.42, N 1.09, Br 37.45.
- Found, No. 54. " 2.37, " 1.12, " 35.33.

The residue, formed on concentrating the alcoholic mother liquor, analyzed as follows:

- No. 56. 0.3050 gm. substance: 0.0162 gm.  $Mg_2P_2O_7$ .  
 0.1968 " " required (Kjeldahl) 1.05 cc. 0.1 N HCl.  
 0.1780 " " (Carius) 0.1334 gm. AgBr.
- Found, No. 56. P 1.48, N 0.74, Br 31.89.

*Hydrolytic Products of the Octabromolecithin.*—The fractions analyzing as octabromolecithin, Nos. 51 and 52, were combined and hydrolyzed by boiling for 8 hours with 10 per cent hydrochloric acid. The fatty acids were removed by filtration and thoroughly extracted with ether. The ether-insoluble acid (5 gm.) purified by extraction with boiling benzene and boiling

glacial acetic acid, when heated for a melting point, decomposed at 243°C. after preliminary darkening. It analyzed as follows:

0.1048 gm. substance:	0.0978 gm. CO <sub>2</sub> and 0.0316 gm. H <sub>2</sub> O.
0.1052 " "	(Carius) 0.1680 gm. AgBr.
Calculated. Octabromoarachidonic acid, C <sub>20</sub> H <sub>32</sub> O <sub>2</sub> Br <sub>8</sub> .	
	C 25.43, H 3.42, Br 67.72.
Found. " 25.44 " 3.37 " 67.96.	

The ether-soluble acids were esterified with methyl alcohol, and the methyl esters fractionally distilled at 0.5 mm. The boiling point ranged from 145–180°C. Four fractions were distilled of which the highest and the lowest, after saponification and conversion to the free acid, gave the following analyses, molecular weights, and melting points.

No. 96. 0.1005 gm. substance:	0.2772 gm. CO <sub>2</sub> and 0.1146 gm. H <sub>2</sub> O.
0.5734 " "	required 22.1 cc. 0.1 N NaOH for neutralization.

It melted at 62.5–63°C.

No. 95. 0.1006 gm. substance:	0.2812 gm. CO <sub>2</sub> and 0.1152 gm. H <sub>2</sub> O.
0.4299 " "	required 15.2 cc. 0.1 N NaOH for neutralization.

It melted at 70°C.

Calculated. C <sub>16</sub> H <sub>22</sub> O <sub>2</sub> .	C 74.92, H 12.58, mol. wt. 256, m.p. 63–64°.
Found, No. 96. " 75.18, " 12.75, " " 259, " 63–64°.	
Calculated. C <sub>18</sub> H <sub>26</sub> O <sub>2</sub> .	" 75.98, " 12.76, " " 284, " 70–71°.
Found, No. 95. " 76.22, " 12.81, " " 283, " 70°.	

*Hydrolytic Products of the Hexabromolecithin.*—Nos. 54 and 55 (25 gm.), the fractions analyzing as a hexabromolecithin, were combined and hydrolyzed with 10 per cent hydrochloric acid. The fatty acids were removed by filtration. A small amount of ether-insoluble material was removed but was not sufficient in quantity for any adequate identification. The soluble material was dried and concentrated. The addition of methyl alcohol to form the ester caused the precipitation of a small amount of material which melted at 145°. It was further purified by solution in benzene and precipitation by gasoline. It then melted at 180°C. and analyzed as follows:

No. 67. 0.1043 gm. substance:	0.1124 gm. CO <sub>2</sub> and 0.0392 gm. H <sub>2</sub> O.
0.1303 " "	(Carius) 0.1946 gm. AgBr.
Calculated. C <sub>18</sub> H <sub>30</sub> O <sub>2</sub> Br <sub>6</sub> . C 28.49, H 3.99, Br 63.26.	
Found, No. 67. " 29.38, " 4.20, " 63.56.	



The bulk of the acids was esterified with methyl alcohol. The esters were fractionally distilled by the usual technique. At 0.2 mm. pressure, the boiling points of the four fractions separated ranged from 150–180°C. The residue of partly brominated materials was not further purified. The lowest and highest fractions, converted to the free acids, gave the following melting points, molecular weights, and analyses:

No. 93. 0.1004 gm. substance: 0.2824 gm. CO<sub>2</sub> and 0.1166 gm. H<sub>2</sub>O.  
 0.5073 “ “ required for neutralization 19.6 cc. 0.1  
 N NaOH.

It melted at 63–64°.

No. 100. 0.1000 gm. substance: 0.2810 gm. CO<sub>2</sub> and 0.1166 gm. H<sub>2</sub>O.  
 0.4011 “ “ required for neutralization 14.2 cc. of 0.1  
 N NaOH.

It melted at 69.5–70°C.

Calculated. C<sub>18</sub>H<sub>32</sub>O<sub>2</sub>. C 74.92, H 12.58, mol. wt. 256, m.p. 63–64°.

Found, No. 93. “ 74.85, “ 12.60, “ “ 258, “ 63–64°.

Calculated. C<sub>18</sub>H<sub>30</sub>O<sub>2</sub>. “ 75.98, “ 12.76, “ “ 284, “ 70–71°.

Found, No. 100\*. “ 76.63, “ 13.04, “ “ 282, “ 69.5–70°.

*Bromination of Egg Lecithin.*—Egg lecithin was prepared from the ether extract of fresh eggs by the usual isolation of the amino-free cadmium salt and subsequent decomposition with ammonia. This material had a higher iodine value (47) than that (30 to 35) usual for the lecithin fraction obtained from the desiccated egg powder. It analyzed as follows:

No. 63. 0.1002 gm. substance: 0.2356 gm. CO<sub>2</sub>, 0.0910 gm. H<sub>2</sub>O, and  
 0.0110 gm. ash.

0.0960 gm. substance: 0.0130 gm. Mg<sub>2</sub>P<sub>2</sub>O<sub>7</sub>.

0.0960 “ “ required (Kjeldahl) 1.5 cc. 0.1 N HCl.

0.2632 “ “ absorbed 0.1227 gm. iodine.

Calculated. Oleyl stearyl lecithin.

C 65.55, H 11.01, N 1.73, P 3.85, iodine No. 31.8.

Found, No. 63. “ 64.12, “ 10.16, “ 2.18, “ 3.77, “ “ 47.

This lecithin was dissolved in a minimal quantity of ether and the solution diluted with two volumes of gasoline (b.p. 40–60°). Through it a stream of bromine in carbon dioxide was slowly bubbled at a rate which permitted maintaining the temperature at

\* Owing to lack of material it has not been possible to repeat this analysis and obtain analytical figures more nearly in accord with the molecular weight and melting point.

-5°. No precipitate settled from the solution either on standing or on the addition of gasoline. It was therefore concentrated *in vacuo* to a thick syrup from which the bulk of the brominated lecithin was precipitated by acetone. The material remaining in the mother liquor was precipitated as a finely divided white powder by the addition of an alcoholic solution of cadmium chloride. This salt, which differed from the corresponding cadmium salt of the unsaturated lecithin by its ready solubility in ether, was dissolved in ether and decomposed with methyl alcoholic ammonia. The inorganic precipitate was removed by filtration and the concentrated residue extracted with very small amounts of iced acetone. It analyzed as follows:

No. 81.	0.3000 gm. substance:	0.0348 gm. $Mg_2P_2O_7$ .
	0.2000 " "	required (Kjeldahl) 1.71 cc. 0.1 N HCl.
	0.1154 " "	(Carius) 0.0430 gm. AgBr.
Calculated. Dibromostearyl stearic lecithin.		
	P 3.22, N 1.45, Br 16.56.	
Calculated. Dibromostearyl palmityl lecithin.		
	P 3.30, N 1.49, Br 17.06.	
Found, No. 81.	" 3.23, " 1.71, " 17.07.	

The acetone-insoluble fraction of the bromolecithin was dissolved in absolute alcohol from which a small amount of a viscous oil separated on standing. This analyzed as follows:

No. 80.	0.3000 gm. substance:	0.0276 gm. $Mg_2P_2O_7$ .
	0.2000 " "	required (Kjeldahl) 1.57 cc. 0.1 N HCl.
	0.1792 " "	(Carius) 0.0946 gm. AgBr.
Found, No. 80.	P 2.56, N 1.57, Br 22.07.	

The alcoholic mother liquor was concentrated *in vacuo* until it foamed. The syrup was precipitated with iced acetone, giving two fractions: No. 82 insoluble, and No. 84 soluble in acetone. The latter was reclaimed by evaporation of the acetone. Their respective analyses were:

No. 82.	0.3000 gm. substance:	0.0246 gm. $Mg_2P_2O_7$ .
	0.2000 " "	required (Kjeldahl) 1.50 cc. 0.1 N HCl.
	0.1194 " "	(Carius) 0.0868 gm. AgBr.
No. 83.	0.3000 " "	0.0298 gm. $Mg_2P_2O_7$ .
	0.2000 " "	required (Kjeldahl) 2.00 cc. 0.1 N HCl.
	0.1410 " "	(Carius) 0.0948 gm. AgBr.

Calculated. Tetrabromostearyl stearic lecithin.

P 2.76, N 1.24, Br 28.50.

Found, No. 82. " 2.28, " 1.05, " 30.94.

" 83. " 2.77, " 1.40, " 28.68.

12 gm. of tetrabromolecithin were hydrolyzed with 10 per cent hydrochloric acid. The mixed fatty acids were fractionated by pouring the ethereal solution into gasoline. The precipitated acid melted at 113–114° and analyzed as follows:

No. 57. 0.1070 gm. substance: 0.1412 gm. CO<sub>2</sub> and 0.0500 gm. H<sub>2</sub>O.

0.1282 " " (Carius) 0.1614 gm. AgBr.

Calculated. Tetrabromostearic acid.

C 36.01, H 5.38, Br 53.28, m.p. 113–114°.

Found, No. 57. " 35.99, " 5.22, " 53.58, " 113–114°.

The soluble acids were converted to the methyl esters and the mixed esters were fractionally distilled at 0.2 mm. pressure. Three fractions were separated distilling from 145–185°C. After conversion to the free acid, and further purification through the lead salt, these melted respectively at 63–64°, 57–58°, and 70–71°. Their analyses and molecular weights were:

No. 71 (96). 0.1005 gm. substance: 0.2772 gm. CO<sub>2</sub> and 0.1146 gm. H<sub>2</sub>O.

0.3027 " " required for neutralization 11.60 cc.

0.1 N NaOH.

0.2263 gm. substance required for neutralization 8.45 cc. 0.1

N NaOH.

No. 73 (92). 0.1003 gm. substance: 0.2786 gm. CO<sub>2</sub> and 0.1116 gm. H<sub>2</sub>O.

0.3960 " " required for neutralization 10.3 cc.

0.1 N NaOH.

Calculated C<sub>16</sub>H<sub>32</sub>O<sub>2</sub>. C 74.93, H 12.58, mol. wt. 256, m.p. 64°.

" C<sub>18</sub>H<sub>36</sub>O<sub>2</sub>. " 75.98, " 12.72, " " 284, " 70–71°.

Found, No. 71. " 75.18, " 12.75, " " 260, " 63–64°.

" 73. " 75.74, " 12.45, " " 284, " 70–71°.

## THE PHOSPHORUS CONTENT OF THE BODY IN RELATION TO AGE, GROWTH, AND FOOD.\*

BY H. C. SHERMAN AND E. J. QUINN.

(From the Department of Chemistry, Columbia University, New York.)

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A previous paper from this laboratory<sup>1</sup> has dealt with the results of an extended series of determinations of calcium in the bodies of experimental animals (rats) and especially with the relation of the calcium content of the body to the age, growth, and food of the animal. The white rat was selected as the experimental animal for this study because of its omnivorous food habits, the close resemblance of the chemical processes of human and rat metabolism, the convenient size of the rat for investigations of this character, and the fact that many features of the nutrition and life history of the rat have been extensively studied by numerous investigators.

The constantly increasing recognition of the importance of phosphorus in nutrition, and especially of the intimate connection between calcium and phosphorus in the development of the growing bone, has led us to extend our studies of the mineral elements in food and nutrition to an investigation of the phosphorus content of the body in relation to age, growth, and food, on a plan similar to that followed in our previous studies of the calcium content of the body. The purpose of the present paper is to summarize the results of our analyses with respect to phosphorus and to point out certain relationships between the calcium and the phosphorus content of the body at different stages of growth and development, and as influenced by differences in the food supply and by the nutritional demands of reproduction and lactation.

As in the case of the corresponding study of calcium,<sup>1</sup> the albino rat was used as experimental animal and each animal analyzed was of definitely known family and nutritional history. In all cases

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<sup>1</sup> Sherman, H. C., and MacLeod, F. L., *J. Biol. Chem.*, 1925, lxiv, 429.

dealt with in this paper the food of the experimental animals and of their mothers had consisted of weighed proportions of food materials so finely ground and so thoroughly mixed as to ensure that the actual food intake of each animal was of accurately known composition at all times. The animals were given food and distilled water *ad libitum*; and to prevent any possible consumption of other material they were kept in all-metal cages without bedding.

From among animals raised in this manner, specimens taken for analysis at the different desired ages were chloroformed and (in all cases except those less than 28 days of age) the contents of the digestive tract removed, weighed, and rejected. The gross weight of the animal minus the weight of the contents of the digestive tract was taken as the net body weight.

For the determination of phosphorus, the body of the rat was placed in a silica dish, charred slowly over a free flame, and burned at a dull red heat in an electric muffle to a white or light gray ash. The ash was dissolved with nitric or hydrochloric acid, the solution filtered and made up to volume. An aliquot portion of this solution was then withdrawn, diluted, heated on a steam bath for several hours and until the solution was evaporated to from 30 to 50 cc. This solution was then employed for the determination of phosphorus by the method of double precipitation, first as ammonium phosphomolybdate and then as ammonium magnesium phosphate, with final weighing of magnesium pyrophosphate, all essentially as described in the Methods of Analysis of the Association of Official Agricultural Chemists.

#### *Phosphorus Content of Normal Rats at Different Ages.*

Since previous work in this laboratory had shown that our Diet B (Laboratory No. 13), a dry food mixture consisting of one-third whole milk powder and two-thirds ground whole wheat, with sodium chloride in the proportion of 2 per cent of the weight of the wheat, served for normal nutrition generation after generation (some families in our colony being now in the seventeenth generation upon Diet B and distilled water only) the analyses of rats raised on this diet are regarded as furnishing values for the normal phosphorus content of the body at different ages; and by far the greater number of the animals used for the determination of these

normal values were raised on this diet. As in the case of our similar investigation of the calcium content of the body,<sup>1</sup> we have confirmed the choice of animals from this diet for the establish-

TABLE I.  
*Average Phosphorus Content of Normal Male Rats.*

Age. <i>days</i>	No. of cases.	Average net weight. <i>gm.</i>	Phosphorus in body.		Probable error of per cent.	Coefficient of variation of per cent.
			<i>gm.</i>	<i>per cent</i>		
At birth.	21	4.3	0.0147	0.34	±0.007	14.34
15	31	21.2	0.1032	0.49	±0.003	5.00
28	24	51	0.268	0.53	±0.005	6.53
61	34	142	0.814	0.57	±0.002	2.47
92	18	226	1.399	0.62	±0.005	5.35
119	8	253	1.633	0.65	±0.004	2.67
241	12	304	2.076	0.68	±0.005	3.90
459	12	301	2.138	0.71	±0.009	6.45

TABLE II.  
*Average Phosphorus Content of Normal Female Rats.*

Age. <i>days</i>	No. of cases.	Average net weight. <i>gm.</i>	Phosphorus in body.		Probable error of per cent.	Coefficient of variation of per cent.
			<i>gm.</i>	<i>per cent</i>		
At birth.	21	4.3	0.0147	0.34	±0.007	14.34
15	18	19.7	0.0959	0.49	±0.003	4.09
28	30	44	0.248	0.56	±0.005	7.36
61	33	108	0.704	0.65	±0.004	5.75
90	14	160	1.091	0.68	±0.006	5.09
120*	7	188	1.296	0.69	±0.006	3.55
235*	10	213	1.573	0.74	±0.007	4.49
154†	4	192	1.268	0.66	±0.018	8.30
267†	15	211	1.419	0.67	±0.007	6.17
559†	15	226	1.704	0.75	±0.008	6.67

\* Adult females which had borne no young.

† Adult females which had raised young.

ment of our norm by analyzing a number of rats which had been fed upon slight modifications of this diet and finding that these gave essentially the same results. Hence we conclude that this diet, while it does not give the greatest rate of growth which we are

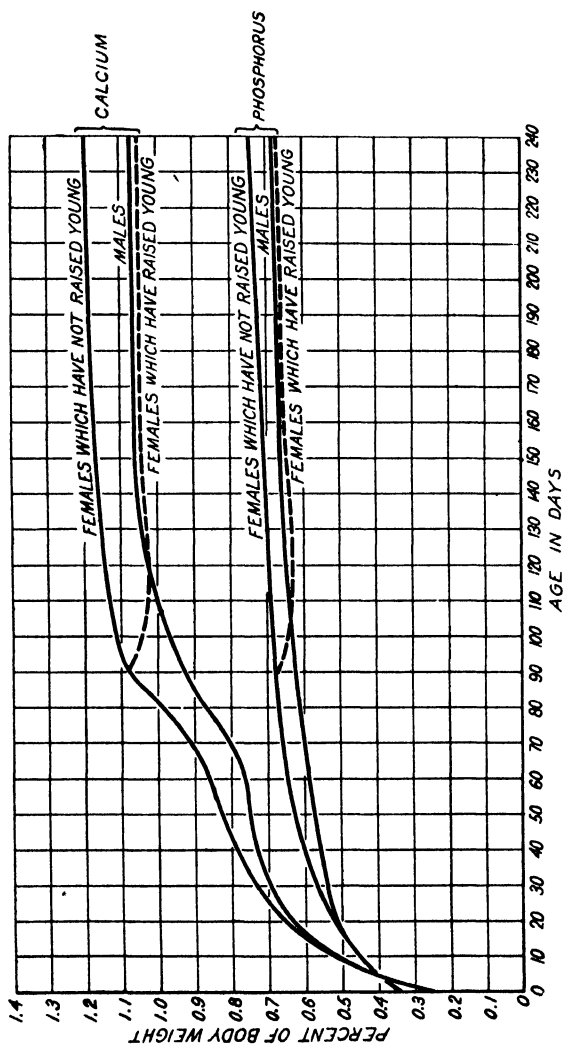


Fig. 1. Changes in the percentages of calcium and phosphorus with age, as shown by analyses of normal albino rats.

able to induce, is well within the range of what should be considered the normal.

Animals were taken for analysis at birth, at 15, and at 28 days, at approximately 60, 90, and 120 days, and at intervals throughout adult life. For purposes of tabulation and charting, all the individuals of the same age, or of a given range of age, are here averaged. The average data are given in Tables I and II and smoothed curves showing the trend of the results in comparison with the corresponding data for calcium are shown in Figs. 1 and 2.

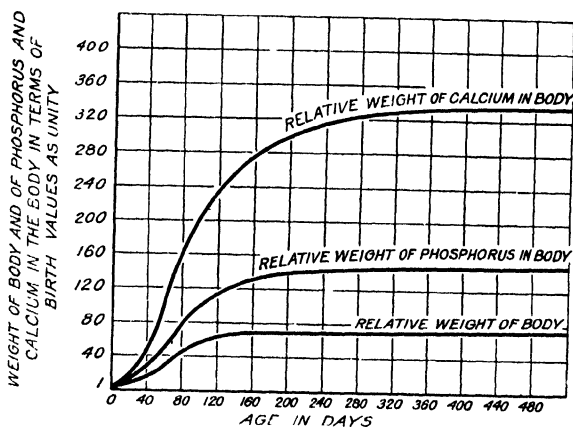


FIG. 2. Relative gains in body weight and weights of calcium and phosphorus in the body, in terms of birth values as unity. (Based upon analyses of normal white rats.)

Taking the data for phosphorus here reported and those for calcium reported in a previous paper<sup>1</sup> it will be seen that at birth the average rat contains 14.7 mg. of phosphorus or 0.34 per cent of its body weight, as compared with 10.8 mg. or 0.25 per cent of calcium. During growth, the body phosphorus increases in greater ratio than the body weight, but not in such great ratio as does the body calcium. Thus the average adult male has about 70 times the body weight with which he was born, whereas this adult body contains about 150 times as much phosphorus and about 340 times as much calcium as did the body at birth (Fig. 2). During the first 15 days of life the rat increases his body weight



about fivefold, his body phosphorus about sevenfold, his body calcium about twelvefold. This brings the average male rat at the age of 15 days to a body content of 0.49 per cent phosphorus and 0.58 per cent calcium. Thus well within the suckling period the percentage of calcium overtakes and passes the percentage of phosphorus in the body. The percentages of both elements continue to increase steadily throughout growth, the calcium more rapidly than the phosphorus, as is readily seen by a comparison of the corresponding curves for the two elements in Fig. 1.

As was found in the corresponding study of calcium,<sup>1</sup> the females with their less rapid growth and lesser adult size than the males, show smaller amounts but higher percentages of phosphorus; except that the bearing and suckling of young causes a loss of phosphorus as well as of calcium from the body. This results in lowering the average percentages of these elements in the bodies of females which have raised young to a level very slightly below that of males of the same age which have had the same food.

Omitting the females which have raised young, the average percentages of phosphorus found at successive ages beyond the suckling period are: at 28 days, males 0.53, females 0.56 per cent; at 61 days, males 0.57, females 0.65 per cent; at 90 to 92 days, males 0.62, females 0.68 per cent; at 119 to 120 days, males 0.65, females 0.69 per cent; in young adults (about 8 months old), males 0.68, females 0.74 per cent.

It is noteworthy that whether we consider the gains during growth, the comparison of males and females, or the effect upon the females of the bearing and suckling of young, we find that the percentages of phosphorus in the body always show the same trend as those of calcium, though the differences are less pronounced. Undoubtedly this is because the differences in both cases are attributable chiefly to the changing proportion of calcium phosphate in the bones, and to the further fact that this (the calcium phosphate of the bones) constitutes a larger proportion of the total calcium than of the total phosphorus of the body. In the rat the bones contain relatively little calcium phosphate at birth but calcification proceeds rapidly during infancy and continues more gradually throughout the whole period of growth and into early adult life.

It is interesting to consider the changes in the calcium and phos-

phorus contents of the body not only in terms of percentages, but also of atomic ratios.

The new born rat with its undeveloped bones contains, in its body as a whole, about three atoms of calcium to five atoms of phosphorus. During the suckling period, while there is a considerable increase of soft tissue which is presumably of about the same composition as that in the new born, the largest gains of calcium and phosphorus are due to the deposition of tricalcium phosphate in the bones. As tricalcium phosphate contains three atoms of calcium to two of phosphorus the rapid gain of this substance must tend to increase the ratio of calcium to phosphorus in the body as a whole, and correspondingly we find that the ratio which was three atoms of calcium to five of phosphorus in the new born, has before the end of the suckling period become a ratio of approximately one to one. Then for some time the calcification of the bones and the growth of the body as a whole appear to run about parallel so that the body retains about equal numbers of atoms of calcium and phosphorus and the atomic ratio of the two elements remains at about one to one. Thereafter a relatively earlier or more pronounced slackening of the general growth than of the calcification of the skeleton results in another (gradual) increase in the ratio of calcium to phosphorus, up to about six atoms of calcium to five of phosphorus in the body of the fully mature adult. This last ratio corresponds with the general belief that the skeleton contains about 99 per cent of the calcium and nearly four-fifths of the total phosphorus of the adult body.

The evidence, afforded by our analyses, of a more rapid calcification in females than in males coordinates well with the observation recently reported by Hammett<sup>2</sup> of a higher water content in the bones of males than of females of the same age.

The bearing and suckling of young causes a loss of body phosphorus coincident with that of body calcium<sup>1</sup> but less pronounced in degree. The tendency to regain the mineral elements thus lost is also observable about proportionately for the two elements. It seems probable that these losses occur primarily because of the drain of lactation upon the mother's body calcium, causing losses from the bones since there is little other available store of calcium in the body, and that the withdrawal of calcium phosphate from

<sup>2</sup> Hammett, F. S., *J. Biol. Chem.*, 1925, lxiv, 409.

the bones to furnish material for milk production results in simultaneous losses of calcium and phosphorus in about the proportions in which they exist in the calcium phosphate of the bones.

This general conception of the gains and losses of phosphorus, as largely dependent upon deposition or depletion of the calcium phosphate of the bones, is strikingly supported by the data obtained in analyzing for phosphorus the animals which had previously been analyzed for calcium after having been kept upon a diet somewhat low in calcium with or without the addition in some cases of cod liver oil and in other cases of calcium lactate. These are described in the section which follows.

*Phosphorus Content of Rats on a Diet Adequate According to Current Standards but of Less Than Optimal Calcium Content, with and without Dietary Supplements.*

The experience of this laboratory has shown<sup>3</sup> that a mixture of one-sixth whole milk powder and five-sixths ground whole wheat with table salt (Diet A, Diet 16) is adequate according to current standards since it is able to meet all the nutritional needs not only of growth but also of reproduction and lactation for generation after generation, some families of rats in our colony being now in the fourteenth successive generation on this diet alone. This diet, however, does not induce as good results as are obtained with Diet B which contains a higher proportion of the whole milk powder and which in chemical terms is richer in calcium and in fat-soluble vitamin. On determining the calcium content of animals taken from Diet A at 60 and 90 days of age it was found that this was appreciably below the average for rats taken at the same ages from Diet B<sup>1</sup> and now a similar, though slightly less pronounced, difference is found in the phosphorus content. The results are summarized in Table III. Evidently on Diet A the deposition of calcium phosphate in the growing bones is slightly retarded or at least does not proceed so rapidly as it does on Diet B. Moreover it was found that the data for phosphorus also paralleled those for calcium in the bodies of the rats which had received in addition to Diet A a supplement of fat-soluble vitamin in the form of cod liver oil or of calcium in the form of calcium lactate. The addition of

<sup>3</sup> Sherman, H. C., and Campbell, H. L., *J. Biol. Chem.*, 1924, 1x, 5.

the fat-soluble vitamins of cod liver oil to this diet had no appreciable effect upon the storage of calcium or phosphorus in the body, while the addition of calcium resulted in an increased storage of both calcium and phosphorus.

Evidently the "limiting factor" in these cases was the low calcium content of Diet A which retarded the deposition of calcium phosphate in the bones even when extra "calcification vitamin" was supplied by adding cod liver oil to the diet. Because of the shortage of calcium and consequent slowing of the deposition of calcium phosphate, the normal increases both of the calcium and of the phosphorus content of the body were retarded; when extra calcium was supplied, more rapid deposition of calcium and phosphorus was made possible, and, although there had been no in-

TABLE III.

*Average Percentages of Phosphorus in Rats Fed on Diet A and Modifications of Diet A.*

Age.....	Males.		Females.	
	60 days.	90 days.	60 days.	90 days.
Diet A.....	0.51	0.54	0.52	0.57
" " + 1 per cent cod liver oil.	0.49	0.52	0.51	0.57
" " + 1 " " calcium lactate.....	0.59	0.62	0.59	0.71

crease in the phosphorus content of the food, the phosphorus content of the body was increased.

The converse of this, namely, that a deficiency of phosphorus in food may retard the deposition of calcium phosphate in the bones and thus the storage of both calcium and phosphorus by the body, and that in such case the addition of a phosphate to the diet will induce the increased storage of calcium as well as phosphorus, was demonstrated several years ago in this laboratory<sup>4</sup> and has since been confirmed by many observers in connection with studies of experimental rickets.

It is interesting also to find that notwithstanding the retardation of calcification and therefore of storage of both calcium and phos-

<sup>4</sup> Sherman, H. C., and Pappenheimer, A. M., *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 193.

phorus in the rats on Diet A, they were able, when simply continued upon this same diet, to continue their gradual storage until they had finally (after the period of most rapid growth in body weight) attained the same normal percentage of body phosphorus as was found in the rats which had been raised on Diet B and used in establishing the normal averages shown in Table I.

#### SUMMARY.

Large numbers of normal white rats of known heredity and nutritional history have been analyzed in order to establish the normal phosphorus content for each sex at various stages of growth and development.

The average percentage of phosphorus in the body of the normal white rat is found to increase from 0.34 per cent at birth to about 0.49 per cent at 15 days; 0.53 to 0.56 per cent at 28 days; 0.57 to 0.65 per cent at 61 days; 0.62 to 0.68 per cent at 3 months; 0.65 to 0.69 per cent at 4 months; 0.70 to 0.75 per cent in adult life.

At the age of 15 days and thereafter, the total weight of phosphorus averaged higher in the males than in the females; but only because of their greater average body weights.

Females which had not borne young showed higher percentages of phosphorus than males of the same age and inheritance and of the same dietary history.

Females which had borne and suckled young showed lower percentages of body phosphorus than those which had not raised young. Such animals show a tendency to regain at least a part of the phosphorus lost, apparently somewhat in proportion to the length of time which has elapsed since the last lactation period.

In all of these respects the variations in the phosphorus content of the body show similar relations to the variations in calcium content as reported in a previous paper from this laboratory.

That the gains and losses of calcium and phosphorus, while not arithmetically parallel, are chemically interdependent is further attested by determinations of calcium and phosphorus in animals which had received a diet adequate according to current standards but low in calcium content, with and without supplements of cod liver oil or of calcium lactate. Here it was found that the calcium intake was a limiting factor as regards storage of both calcium and

phosphorus in the body, and that while under the conditions of this investigation cod liver oil did not increase the storage of either of these elements, the storage of both calcium and phosphorus was increased when calcium only was added to the food.

In some respects the results become clearer when considered in terms of the relative proportions of calcium and phosphorus atoms in the body as a whole and in the calcium phosphate of the bones.

In whatever terms expressed, it is striking to find that whereas the body of the rat, in making a normal growth and development from birth to maturity must multiply its original weight by about 70, it must multiply its original phosphorus by about 150, and its original calcium by about 340.

The quantitative relations for all ages from birth to middle age are shown graphically. The data are offered both as a contribution to the fuller understanding of the chemistry of normal growth and development and as a basis for the interpretation of experiments in which the development of the body as a whole, or of the bones specifically, is retarded by dietary deficiency or other abnormality of nutrition.



## CONCERNING THE RELATION OF GUANIDINE TO PARATHYROID TETANY.

By J. B. COLLIP AND E. P. CLARK.

(From the Department of Biochemistry, University of Alberta, Edmonton,  
Alberta, Canada.)

(Received for publication, January 5, 1926.)

### INTRODUCTION.

The discovery that the parathyroid hormone can be successfully used to control or prevent tetany (1) in parathyroidectomized dogs is in itself neither proof nor disproof of the guanidine intoxication theory of tetany (2). It occurred to us to put the guanidine theory to a severe test by following the urea and non-protein nitrogen curves of the blood of untreated parathyroidectomized dogs and ascertaining if such curves bear any relationship to similar curves obtained following the injection of certain guanidine compounds. It was thought that intoxication by guanidine compounds should manifest itself by an absolute increase in the non-protein nitrogen of the blood and also by a change in the ratio between the non-protein and the urea nitrogen.

Salvesen (3) found no increase in the non-protein nitrogen in parathyroid tetany. Our results are in accord with this finding, with the exception that immediately preceding death a definite increase in non-protein and urea nitrogen may be noted.

### *Methods.*

Dogs were used. Thyroparathyroidectomized animals were bled from a leg vein prior to operation and again at intervals thereafter. The animals used in the guanidine experiments were bled in a similar manner both prior to the subcutaneous injection of the guanidine compound and at frequent intervals subsequent to the injection. Urea was estimated according to the technique recently developed in this laboratory and non-protein nitrogen was determined by the Pregl micro Kjeldahl procedure (4).



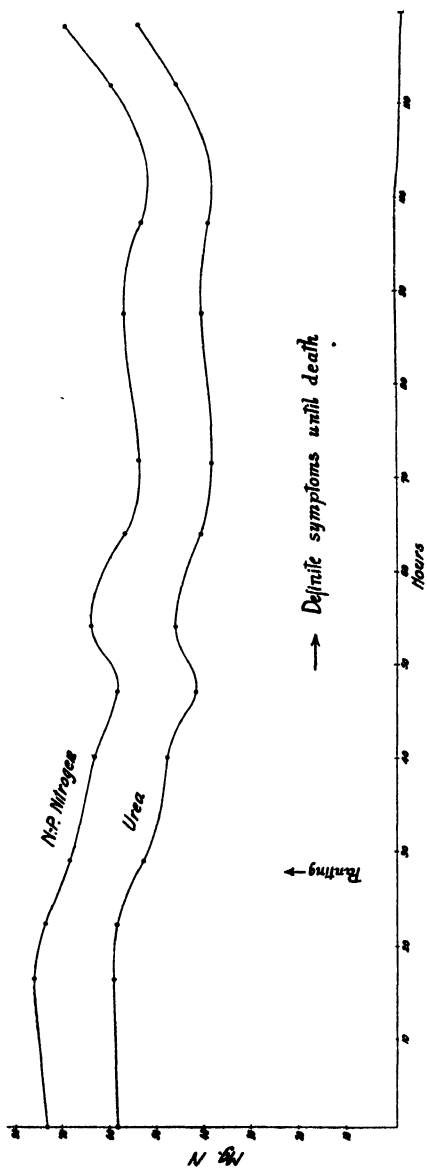


CHART 1. Dog 355, 20.5 kilos. Thyroparathyroidectomy was performed. No treatment was administered. Death resulted in 54 days.

**Results.**—The results are shown graphically in the charts and in the text.

#### DISCUSSION OF RESULTS.

Charts 1, 2, and 3 illustrate the urea and non-protein nitrogen curves obtained on untreated thyroparathyroidectomized dogs.

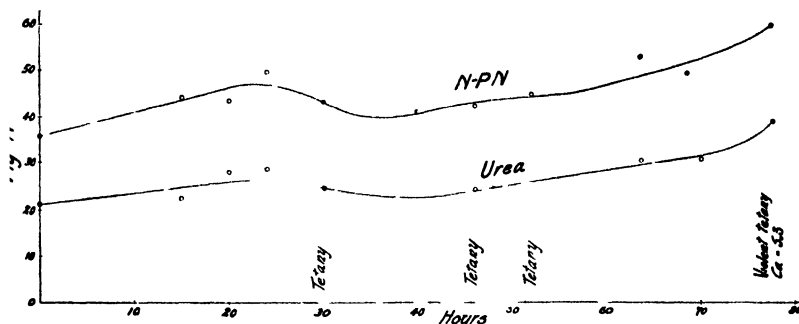


CHART 2. Dog 349, 16 kilos. Untreated thyroparathyroidectomized dog. Last blood sample taken when animal was in state of profound tetany. Parathyroid hormone administered at this point and experiment discontinued.

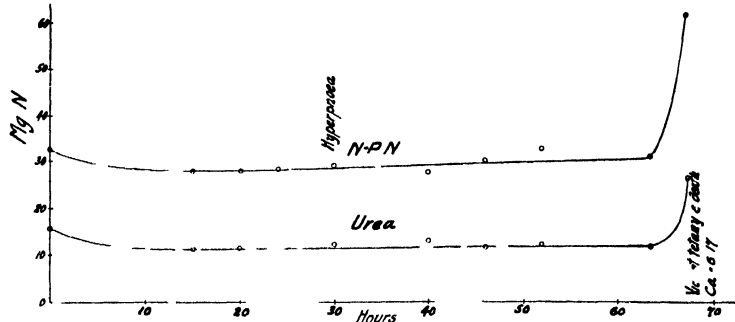


CHART 3. Dog 348, 24 kilos. Untreated thyroparathyroidectomized animal. Last blood sample taken from heart immediately after death.

It will be noted while there is a certain slight fluctuation in the urea and non-protein nitrogen values, that the curves are almost parallel throughout an experiment.

A very large dose of guanidine hydrochloride was administered in the experiment shown in Chart 4. Prostration and muscular

tremors developed in  $2\frac{1}{2}$  hours. The deviation of the urea and non-protein nitrogen curves is indicative of the absorption of the injected guanidine.

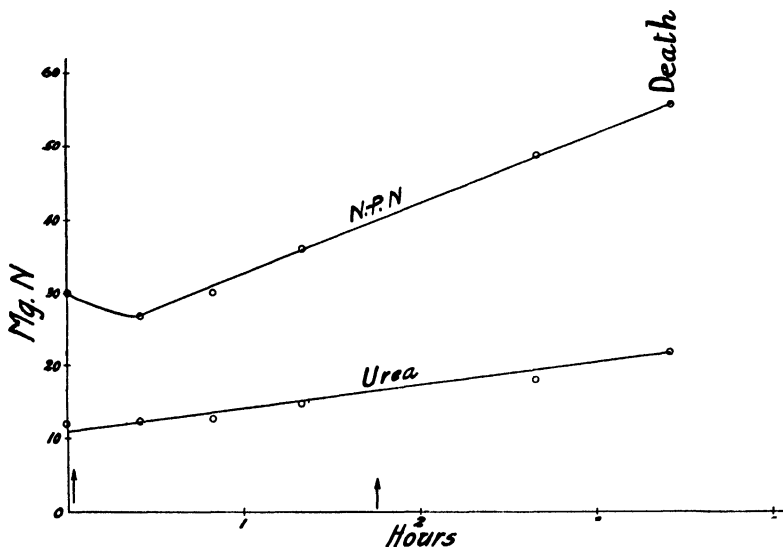


CHART 4. Dog 353, 19 kilos. Guanidine hydrochloride administered. 5 gm. were injected at the beginning of the experiment, and 3.5 gm. after  $1\frac{1}{2}$  hours.

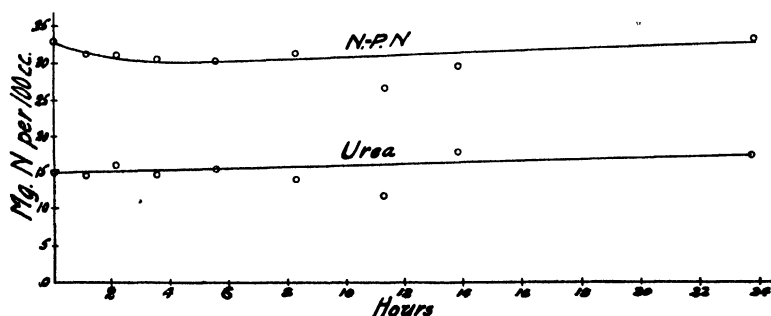


CHART 5. Dog 352, 17 kilos—very fat. Non-effect of subcutaneous injection of 2.6 gm. of guanidine as carbonate.

The experiments illustrated in Charts 5 and 6 are of interest in that the same animal was used in each instance and in each

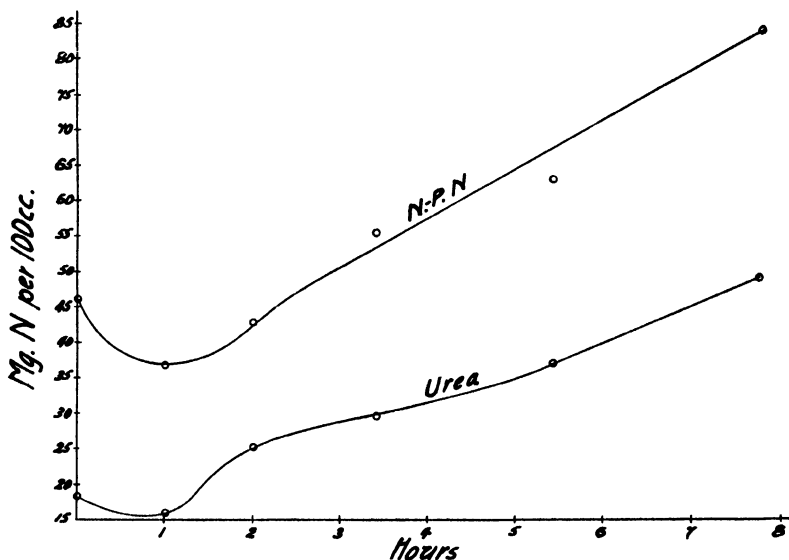


CHART 6. Dog 352, 17 kilos. 2.6 gm. of guanidine as carbonate were injected. Violet tetany developed.

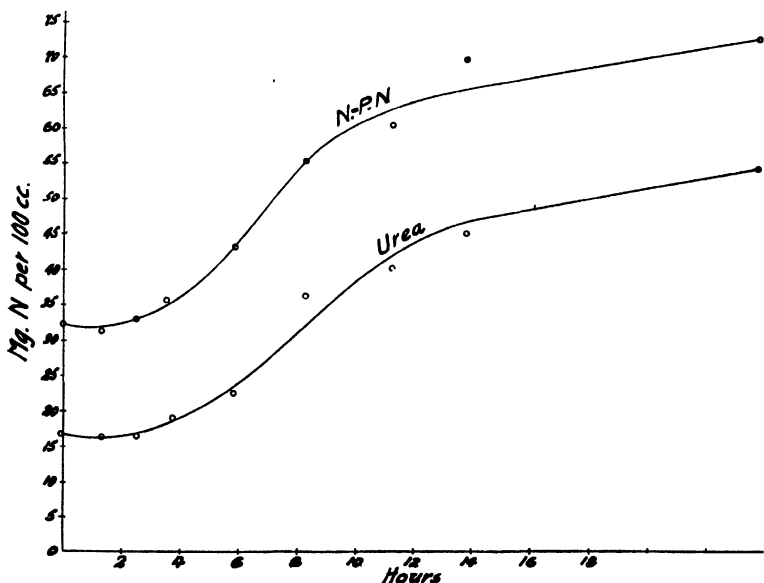


CHART 7. Dog 368, 15.5 kilos. 2.3 gm. of guanidine as carbonate injected. Animal died on the 2nd day. Muscle tremors and spastic gait were noted during last 10 hours of experiment as illustrated on chart.

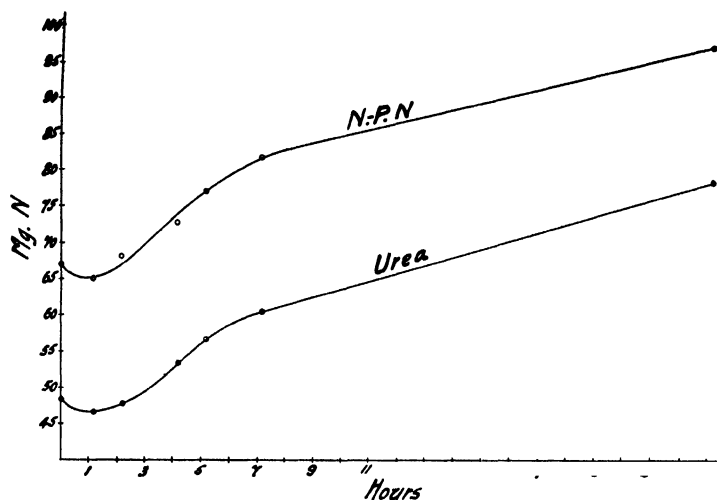


CHART 8. Dog 374, 20 kilos. 2.0 gm. of methylguanidine as sulfate were injected. After 4 hours muscular tremors were noted. At 5.3 hours, half a gm. of methylguanidine was injected. Mild tetany was present at the end of the experiment. Death occurred during the following night.

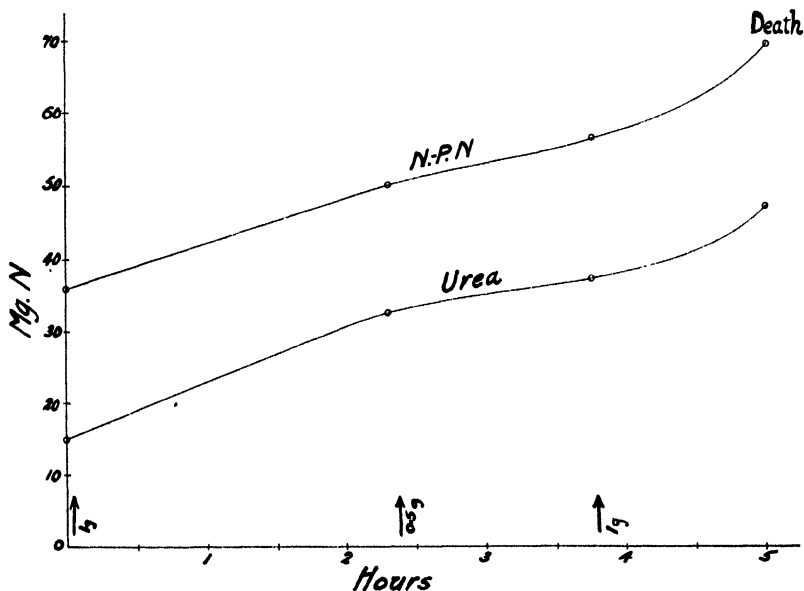


CHART 9. Dog 359, 20 kilos. 2.5 gm. of dimethylguanidine as sulfate administered subcutaneously in divided doses as shown on the chart. Death resulted from tetany.

case 150 mg. of guanidine carbonate per kilo of body weight were injected. No change in the blood chemistry occurred in the first experiment, neither was there any sign of tetany manifested.

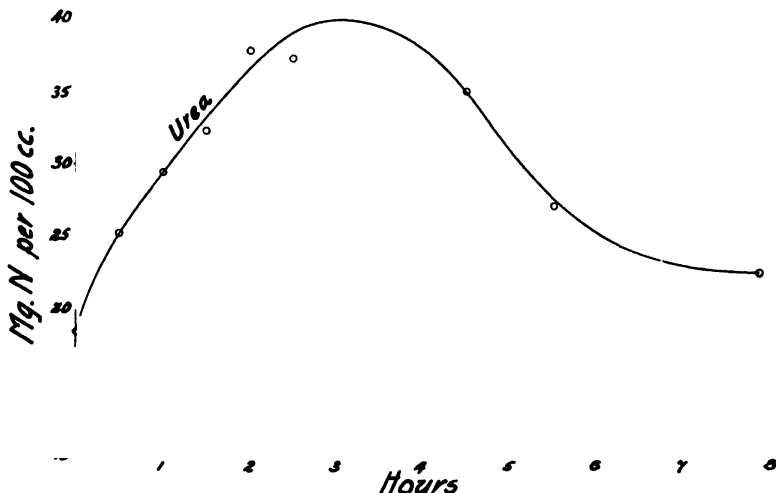


CHART 10. Dog 376, 8 kilos. Effect of the subcutaneous injection of 4 gm. of urea.

Mg. of Urea per 100cc Blood

Hours

CHART 11. Dog 376, 8 kilos. Effect of ingestion of 4 gm. of urea on a fasting stomach.

2 days later the same animal receiving the same treatment developed violent tetany and showed a definite change in the blood chemistry. The result shown in Chart 7 is of the same order.

The effect of the injection of 100 mg. per kilo of body weight of methylguanidine as sulfate is shown in Chart 8, while in Chart 9 the result of the injection of dimethylguanidine sulfate is demonstrated.

It is our opinion that these results show quite conclusively that guanidine tetany (*i.e.*, tetany produced by the guanidine compounds which have been used in these experiments) is of a different order from parathyroid tetany. The nature of the curves in these experiments suggests (1) that injected guanidine may be converted in part into urea; (2) that there is either increased urea formation or else retention due to a toxic action upon the kidney when certain guanidine compounds are administered.

The injection or ingestion of urea causes a different response in the blood urea, from that following the injection of guanidine compounds. See Charts 10 and 11.

It is evident that the above results are not in accord with the guanidine intoxication theory of parathyroid tetany. They do not constitute final proof against the validity of the theory, however, since certain guanidine derivatives might have a much greater toxicity than the compounds used, and it may be argued that cumulative action might cause a different response in the blood chemistry.

Although we have repeatedly tried to discover some specific effect of guanidine injection upon blood calcium, our results have been negative. Inorganic phosphorus has also been followed in many experiments. In the absence of tetany little or no change has been noted in blood phosphorus. In guanidine tetany there was a very definite increase in inorganic phosphorus as others have previously observed (3).

#### SUMMARY.

The urea and non-protein nitrogen curves of untreated parathyroidectomized dogs have been determined.

Similar curves have been obtained following the injection of certain guanidine compounds.

The results obtained are opposed to the guanidine theory of parathyroid tetany.

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# STUDIES ON THE INORGANIC COMPOSITION OF BLOOD.

## I. THE EFFECT OF HEMORRHAGE ON THE INORGANIC COMPOSITION OF SERUM AND CORPUSCLES.

By STANLEY E. KERR.

*(From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia.)*

(Received for publication, January 22, 1926.)

### INTRODUCTION.

The recent development of methods of analysis for the inorganic constituents of blood, requiring only small quantities of material, has permitted investigation of normal and pathological metabolism of inorganic salts. The fact that blood volume is restored after hemorrhage by the inflow of body fluids, presumably differing in inorganic composition from plasma, suggested that the study of inorganic salt metabolism after hemorrhage should yield information of value. The disturbances in the acid-base relationships after hemorrhage, including changes in the alkaline reserve, also make evident the need for more information concerning the variations in the inorganic ions other than bicarbonate. An investigation was therefore undertaken to determine what changes take place in the inorganic composition of the blood of dogs after single and repeated hemorrhages.

The inorganic composition of plasma differs so widely from that of corpuscles that analyses of whole blood without reference to cell composition and the relative proportion of cells and serum are of little significance. This becomes especially true in a series of analyses of the blood from one animal, because of the decrease in cell volume which results from repeated withdrawals of blood. Although the red blood corpuscles are generally regarded as impermeable to the cations, the evidence is insufficient to warrant the assumption that changes do not occur in the sodium and potassium concentrations in the corpuscles. The necessity of studying

plasma and cells as separate systems has not, however, been recognized in much of the work on the inorganic composition of the blood. In the following experiments on the effect of hemorrhage, serum and corpuscles were separately analyzed.

### *Methods.*

Dogs were used because of the large quantities of blood required for analysis. Blood for analysis or for hemorrhage was always obtained from the heart, without anesthesia, by aspirating the blood through a No. 16 lumbar puncture needle into a 200 cc. cylindrical separatory funnel. The operation of bleeding causes little discomfort to the animal. In the later experiments cocaine (10 mg.) was injected subcutaneously at the point to be punctured, with the result that the animal seldom moved or showed the slightest sign of pain. Even when a large quantity of blood was taken, it was found less disturbing to the dog to take the entire quantity rapidly from the heart rather than more slowly from the jugular vein.

The blood was collected under oil and immediately defibrinated by gentle stirring with a glass rod. Defibrination was preferred to the use of anticoagulants because their effects on shifts of water or ions between corpuscles and plasma are unknown. A little fibrin usually fails to cling to the stirring rod used to defibrinate the blood. In order to remove these loose pieces of fibrin, the blood was next filtered through gauze under oil, and received in a 100 cc. glass cylinder containing a layer of oil. The manipulations were carried on without delay so that no opportunity was given for the corpuscles to sediment appreciably. Before sampling, the blood was stirred by rapidly twisting a long glass rod flattened and bent at the lower end to give the effect of a propeller. This permitted efficient mixing of corpuscles and serum with minimum disturbance of the surface and hence minimum loss of  $\text{CO}_2$ . The whole blood was then immediately sampled for chloride and water determinations. A rubber stopper carrying a short inlet tube and a long delivery tube was then inserted into the cylinder. By blowing into the short tube, portions of blood were forced out the delivery tube into the specific gravity bottle, the hematocrit tubes and, under oil, into 50 cc. centrifuge tubes.

*Separation of Serum.*—The oil on the surface of the blood in

the centrifuge tubes was removed as completely as possible and replaced quickly by paraffin just warm enough to be liquid. The tubes were then centrifuged at about 4000 R.P.M. for half an hour. Hematocrit tubes were centrifuged together with the large samples, and the readings found to be constant after half an hour. In the earlier experiments the centrifuging was continued for 1 hour or longer, but this resulted in unnecessary heating of the blood. When removed from the centrifuge the serum was invariably straw-colored and clear, with no trace of hemolysis. The tubes were usually at about body temperature when taken out after half an hour, but hot to the touch when run for an hour. The tubes were cooled in water, the paraffin removed, and the serum aspirated into a small Pyrex Erlenmeyer flask. The final traces of serum were freed from the surface of the corpuscles and the sides of the tube by wiping with strips of filter paper until the corpuscles no longer "wet" the paper. No attempt was made to wash the corpuscles before analysis, for this was found to alter their composition. The corpuscles were analyzed separately for all constituents studied except chlorides which were calculated from the results of analyses on whole blood and serum.

#### *Analytical Methods.*

Water was determined in whole blood, serum, and corpuscles by drying 1 cc. portions at 115–120°C. to constant weight for 24 to 48 hours. The samples were measured quickly onto cones of fluted filter paper contained in small weighing bottles which had been dried for at least 24 hours, cooled, and weighed just previous to use.

The specific gravity of whole blood and serum was determined at room temperature in a 5 cc. pycnometer.

*Weight of Cells: Weight of Blood.*—In order to calculate cell chlorides from mm of Cl per 1000 gm. of whole blood and serum, the percentage of cells by weight rather than volume is needed. The percentage by weight may be calculated from the percentage of water in whole blood, serum, and corpuscles as follows:

Let  $H_2O_w$ ,  $H_2O_s$ ,  $H_2O_c$  represent the percentage by weight of  $H_2O$  in whole blood, serum, and cells. Let per cent. represent the percentage of cells by weight.

Then 100 - per cent<sub>s</sub> represents the percentage of serum by weight.

$100 \text{ H}_2\text{O}_{wb} = \text{H}_2\text{O}_s (100 - \text{per cent}_s) + \text{H}_2\text{O}_c \cdot \text{per cent}_c$ .

$$(\text{Per cent}_c) = \frac{100 (\text{H}_2\text{O}_s - \text{H}_2\text{O}_{wb})}{\text{H}_2\text{O}_s - \text{H}_2\text{O}_c}.$$

The calculated percentages are recorded in the tables under the heading (weight of cells : weight of blood) 100.

The greatest error of the method rests upon the uncertainty of complete separation of serum from the corpuscles. These weight-hematocrit determinations were checked by cell volume determinations made with the Daland hematocrit, and with specially calibrated long hematocrit tubes which were centrifuged together with the blood samples. The volume hematocrit values were on the average 2.6 per cent higher than the weight hematocrit values, indicating that the packing of the cells is not as great in the large specimens as it is in the narrow tubes.

When blood is defibrinated, more serum than corpuscles is taken out with the fibrin, so that the relative volume occupied by cells in defibrinated blood is greater than that in oxalated blood. When there is an increase of fibrin, as after hemorrhage, this difference becomes greater. The hematocrit readings, therefore, give only approximate ideas of the true cell volume in the circulating blood of the animal, but are accurate for calculating cell composition. Only the weight-hematocrit determinations, calculated as indicated above, are given in the tables.

*Chlorides* were determined in whole blood and serum by the method of Van Slyke (1923-24). The pipettes used were those with which the samples for water determination were measured, and thus had been calibrated for delivery of both serum and whole blood by weight. The chloride content of the corpuscles was calculated from chlorides in whole blood and serum by the formula developed as follows.

Let  $\text{Cl}_{wb}$ ,  $\text{Cl}_s$ ,  $\text{Cl}_c$  represent the percentage Cl in whole blood, serum, and corpuscles. Let per cent<sub>c</sub> represent the percentage of cells by weight.

Then  $100 \text{ Cl}_{wb} = \text{Cl}_s (100 - \text{per cent}_c) + \text{Cl}_c \cdot \text{per cent}_c$ .

$$\text{Cl}_c = \text{Cl}_s - \left[ \frac{100 (\text{Cl}_s - \text{Cl}_{wb})}{\text{per cent}_c} \right].$$

*Calcium and magnesium* were determined in serum direct, calcium by the method of Clark and Collip (1925). Magnesium

was determined by the colorimetric method of Briggs (1924) in the serum from which calcium had been removed, together with the washings from the calcium precipitate.

*Serum proteins* were determined by the use of the refractometer, using the methods of calculation of Neuhausen and Rioch (1923) for dog blood.

Sodium, potassium, and inorganic phosphates of both serum and corpuscles were determined in the protein-free filtrates rather than directly in serum or laked corpuscles. Sodium and potassium determinations on serum direct were found unreliable, as will appear in the discussion of these methods. Since it was necessary to remove the proteins from the corpuscles before analysis for sodium and potassium, it was considered best to do the same for serum, handling both alike. The protein-free filtrates were prepared from weighed samples of serum and cells. 20 cc. of serum were weighed in a 100 cc. volumetric flask, diluted with about 50 cc. of water, and the proteins precipitated by the addition of 20 cc. of 20 per cent trichloroacetic acid. After mixing, diluting to the mark, and standing 10 minutes, the contents of the flask were poured into two 50 cc. centrifuge tubes, covered with rubber caps to prevent evaporation, centrifuged for a few minutes, and the almost clear liquid then filtered through ashless filter paper into a Pyrex flask. Due to the much greater proportion of protein in corpuscles than in serum, they were diluted 1:10 instead of 1:5 as for serum. Approximately 15 to 20 gm. of corpuscles were weighed in a 200 cc. volumetric flask, 10 cc. of 0.1 per cent saponin solution and about 100 cc. of water added, the mixture shaken and allowed to stand until hemolysis was complete, as evidenced by the absence of turbidity. One drop of caprylic alcohol was next added to destroy the foam. If this is not done, the protein precipitate occludes many bubbles, and the subsequent dilution to the mark is far from correct. 40 cc. of 20 per cent trichloroacetic acid were next added quickly and the flask rotated and shaken until the precipitation was complete. The precipitate thus formed settled rapidly, and was free from lumps. After all effervescence of gas had ceased, another drop of caprylic alcohol was added and the solution diluted to the mark. After thorough mixing and standing for 10 minutes, the supernatant liquid was filtered through an ashless filter, with precautions to prevent loss

by evaporation, and the filtrate preserved in Pyrex glassware for analysis.

*Inorganic phosphates* were determined in the serum and cell filtrates by Briggs' modification (1924) of the Bell-Doisy method.

*Blood volume* was calculated as 8 per cent of the body weight.

*Potassium*.—The method of Kramer and Tisdall (1921) for the determination of potassium in serum direct was not found to be satisfactory. Determinations on serum direct sometimes agreed with those done on the protein-free filtrate by the method described below, but more often yielded lower results. The sodium cobalti-nitrite reagent precipitates something in serum, probably a protein, which occludes some potassium cobalti-nitrite and prevents its complete oxidation by permanganate. The following procedure for protein-free filtrates, including a modification of the method of washing the precipitate, was found to give accurate results with a solution of known potassium concentration, containing the other inorganic constituents in approximately the concentration found in blood. The method is given in detail, since it differs from that of Kramer and Tisdall in several respects, and because strict adherence to detail is necessary in order to obtain accurate results.

A quantity of protein-free filtrate equivalent to 1 gm. of serum or cells (5 cc. of serum filtrate, or 10 cc. of cell filtrate) is evaporated to complete dryness on the water bath in a platinum dish (or in a small glass beaker). The residue is dissolved in a few drops of water, four or five drops of concentrated  $\text{HNO}_3$  are added, and again evaporated to complete dryness. This procedure removes the trichloroacetic acid more effectively than adding a few drops of  $\text{HNO}_3$  at the beginning of evaporation. Baking on a hot plate should be avoided as the residue cakes. The dry residue is dissolved in about 0.5 cc. of warm water and the solution transferred to a 15 cc. centrifuge tube, rinsing the dish with several small portions of water, the final volume of solution being 2 cc. When cool, 1 cc. of the sodium cobalti-nitrite reagent as prepared by Kramer and Tisdall (1921) is added *drop by drop* from a burette, mixing thoroughly after each drop, and the tube allowed to stand at least 45 minutes. The solution is diluted to 5 cc. with water and mixed by tapping. A small part of the precipitate at this stage and during washing has a tendency to float on the surface

and no amount of centrifuging will throw it down. The addition of 0.5 cc. of a freshly shaken 1 per cent caprylic alcohol-water mixture before centrifuging will prevent the precipitate from floating. After centrifuging for 10 minutes the supernatant liquid is decanted and the tube placed on a pad of filter paper in the inverted position to drain for a few minutes, taking care not to disturb the precipitate by returning the tube to the upright position before draining. The mouth of the tube is wiped out as completely as possible with the tip of a towel, the sides of the tube washed with 5 cc. of water, and the tip of the tube tapped until the precipitate just begins to mix with the water. Caprylic alcohol suspension is added, and the tube is again centrifuged for 10 minutes. After again decanting and draining for 5 minutes, the washing process is once more repeated (two washings in all), and the titration then made with permanganate as in the regular Kramer-Tisdall method. The decantation and draining of the liquid from the precipitate does not cause loss of the precipitate if the centrifuge tubes are carefully selected. The sides of the narrowing conical portion of the tube must be straight, not curved inward, and the tip should not be broad and shallow.

In connection with the titration itself, the best results are obtained when 2 or 3 cc. of permanganate completely oxidize the precipitate. Whenever there is an abnormally large quantity of potassium present the usual addition of permanganate is found insufficient and one must add sometimes 5 cc. to complete the oxidation. In such cases the results will be found to be too high, and the analyses must be repeated taking an aliquot of such size that 2 or 3 cc. of permanganate will oxidize the precipitate. A curve representing the equivalence of cc. of permanganate to mg. of potassium may be constructed rather than relying on the theoretical calculation.

It is evident that these limitations make the method somewhat unsatisfactory, but strict adherence to the procedure described gave good results. Analysis of a solution of salts in the concentration found in blood gave individual results within 4 per cent of the theoretical, while duplicate determinations nearly always averaged within 2 per cent of the theoretical. It is doubtful whether a macro analysis on 10 cc. of serum by any gravimetric procedure would give any greater accuracy than this micro method



where only 1 cc. is used. Controls were run on a solution of blood salts very frequently, and determinations of potassium were repeated when close checks were not obtained.

*Sodium.*—Difficulties were encountered in determining sodium in serum by the method of Kramer and Gittleman (1924–25). A detailed study of this method for sodium gave convincing evidence that far more reliable results could be obtained by the use of the protein-free filtrate. Results obtained on serum direct were often 10 per cent lower than those obtained after removal of the proteins. The following method for sodium was found very satisfactory. It is a combination of the methods of Balint (1924) and Kramer and Gittleman (1924–25) with modifications, and is adaptable to serum, whole blood, or cells.

A protein-free filtrate is prepared from serum or cells as already described. An aliquot of the filtrate equivalent to 2 gm. of serum or cells is measured into a platinum dish and evaporated to dryness on the water bath. The residue is moistened with water, five drops of concentrated  $\text{HNO}_3$  (iron-free) are added, and again evaporated. (If water is not first added, there will be sufficient effervescence to cause loss of some material.) A few drops of concentrated  $\text{HNO}_3$  are now added to the dry residue, and again evaporated. This procedure is repeated once or twice more, and effectively destroys the trichloroacetic acid. The residue is dissolved in about 0.5 cc. of water and transferred to a 50 cc. Pyrex centrifuge tube having a conical bottom, and the dish is rinsed several times until the total volume is 2 cc. A drop of phenolphthalein is added, and the solution made alkaline with a drop or two of 10 per cent KOH (kept in a paraffined bottle). 10 cc. of potassium pyroantimonate, prepared according to the directions of Kramer and Gittleman (1924–25), are next added, and the mixture stirred vigorously with a glass rod, rubbing the sides of the tube until a heavy precipitate forms. If a number of determinations are being done together, the glass rods are numbered to correspond to the tubes. 4 cc. of 80 per cent alcohol are now added from a burette, drop by drop, with constant stirring. The glass rod is rinsed with a small quantity of 30 per cent alcohol contained in a wash bottle. Some of the precipitate remains on the rod. The tube is covered with a rubber cap and allowed to stand for 30 to 45 minutes, not longer. The tube is centrifuged at

moderate speed for 5 minutes, the liquid is decanted and the tube drained in an inverted position for several minutes. After decanting, the tube must be kept in the inverted position, in order to prevent the precipitate from being disturbed. After standing a few minutes, the mouth of the tube is wiped out, and the sides rinsed with 10 cc. of 30 per cent alcohol from a wash bottle. The tube is rotated to wash the precipitate without disturbing it greatly, again centrifuged, the liquid decanted and drained as before. Now each stirring rod is placed in its respective tube, 5 cc. of concentrated HCl (iron-free) are added, dissolving the precipitate adhering to the rod. The packed precipitate is broken up and about 1 cc. of water added. On stirring, the precipitate dissolves instantly, whereas the concentrated HCl will not dissolve it readily. The rod is removed and rinsed with water, and the solution is diluted to 10 cc., mixed, and cooled. About 2 cc. of 2 per cent KI are added, and the liberated iodine is immediately titrated with 0.1 N  $\text{Na}_2\text{S}_2\text{O}_3$  till the color is lemon-yellow. About 0.5 cc. of 1 per cent starch solution is added, and the titration continued very slowly to a colorless end-point. The entire process is thus completed in the same centrifuge tube.

Balint (1924) has shown that the precipitate of pyroantimonate occludes some potassium and the results are about 4 per cent higher than theoretical. Hence it is desirable to standardize the whole procedure against a blood salt mixture rather than to standardize the thiosulfate with great accuracy against iodine or biiodate. The thiosulfate is prepared by dissolving the theoretical quantity (24.832 gm.) in water, diluting to a liter. It is protected from the  $\text{CO}_2$  of the air, and standardized against pure NaCl, or better against a solution of inorganic salts in the concentration found in blood. A series of determinations of sodium is made on 2 cc. portions of such a solution in the manner described. The sodium equivalent of each cc. of thiosulfate is then calculated as follows:

$$\text{Mg. Na in sample taken} \div \text{cc. Na}_2\text{S}_2\text{O}_3 \text{ used} = \text{mg. Na per cc. Na}_2\text{S}_2\text{O}_3.$$

The factor thus obtained should not differ by more than 4 per cent from the theoretical factor 1.15.

Attention should be called to several minor points. (a) The centrifuge tubes and glass rods must be seasoned as described by

Balint by using them in several precipitations of sodium before attempting accurate work. They must be cleaned with water only, never with soap or cleaning fluid. (b) When the reagent fails to precipitate the sodium the fault may be in not having destroyed the trichloroacetic acid properly. If properly conducted, one drop of potassium hydroxide will render the solution alkaline. (c) Traces of dissolved glass will interfere with the precipitation of sodium pyroantimonate. Hence in preparing the pyroantimonate reagent, paraffined funnels and flasks should be used from the time the KOH has been added until the reagent is finally stored in a paraffined bottle. (d) When the end-point of the titration is reached, if the blue color immediately reappears, and continues to do so as more thiosulfate is added, indicating a slow liberation of iodine, the reagent is unsatisfactory and must be discarded.

Kramer and Gittleman (1924-25) recommend adding 3 cc. of 95 per cent alcohol, distilled over KOH. If not distilled, the alcohol forms a precipitate on adding it to the reagent. This also occurs if the alcohol is added too rapidly. 80 per cent alcohol was found satisfactory without redistillation and does not form a precipitate in the reagent as readily as 95 per cent alcohol, by causing too great a concentration of alcohol at the point of addition. A test should, of course, be made to see that no precipitate is formed when 4 cc. of the 80 per cent alcohol are added drop by drop to 10 cc. of the reagent.

This method has given excellent results in the hands of several workers in this laboratory. It is to a certain extent empirical, and details must be strictly adhered to. It has the advantage of eliminating much of the personal equation, since each worker standardizes not only the solutions but the entire procedure on a solution of known sodium content. In fact one cannot rely on sodium determinations unless such controls on a known sodium solution are frequently made.

### *Results.*

The experimental results may be grouped under three heads. (1) The changes in blood 24 hours after a large hemorrhage (Experiments 1-H-A, 1-H-B, and 2-H, Tables I and II). (2) The changes in blood after several days of repeated hemorrhage (Ex-

periments 4-H and 5-H, Tables III and IV). (3) Control experiments—the effect of fasting (Experiments 1-F and 2-F, Tables V and VI).

Experiments 1-H-A and B were conducted on the same dog, 8 days apart. In both cases the dog was offered food, but would not eat for 24 hours after a hemorrhage. In Experiment 2-H the dog fasted before and during the experiment. In these three experiments the hemorrhage was a severe one. The first portion of blood drawn was used for the initial analysis; samples were taken from the animal 4 to 6 hours later, and again 24 hours after the hemorrhage. In Experiments 1-H-A and B the intermediate samples were discarded.

Tables I and II show the absence of any great changes in the inorganic composition of either serum or cells. There is a slight fall in serum sodium in Experiments 1-H-A and B and in 2-H. There is a fall in chlorides in Experiments 1-H-B and 2-H. In the red corpuscles in Experiment 1-H-B there is a decrease in sodium and a rise in potassium following the hemorrhage. These changes are beyond the limits of error of the determinations, and cannot be accounted for by shifts of water. Similar variations, though less pronounced, occurred in Experiment 2-H. It is thus seen that a single hemorrhage produces only a slight fall in sodium and in chlorides in serum, and perhaps a slight fall of sodium and rise of potassium in the corpuscles. In each of these three experiments the fall in hematocrit values indicates that tissue fluids had entered the blood causing a considerable dilution by the end of 24 hours.

Having failed to find any great changes after a single hemorrhage, it was decided to investigate the effects of repeated hemorrhage. Two experiments were conducted in which fasting dogs were bled daily, each hemorrhage being a severe one. In Experiment 4-H on the 4th day of severe hemorrhage, when the bleedings had removed a quantity of blood nearly equal to the original blood volume of the animal, it was found that the sodium and chloride concentrations of the serum had diminished, decreasing in equivalent quantities. A normal value was obtained for serum potassium after repeated hemorrhage. The original potassium level was abnormally low in both serum and cells, but the analyses were repeated, leaving no doubt as to their accuracy. In the cells, sodium was unchanged, but potassium increased greatly in concentration.

TABLE I.  
*Experiment I-H. Effect of Hemorrhage.*  
 ilos. Calculated blood volume 1720 cc. Received water daily.

Sample No.	Date.	Hour.	Volume of blood taken.	Per cent of total blood.	Weight of cells $\times 100$ .	Remarks.	Analyses.	Sample I-A.	Sample II-A.	Sample I-B.	Sample II-B.
I-A	Jan. 19	10 a.m.	320	19	47	Fasted Jan. 18.	{ Whole blood. Serum.	1.061	1.051	1.049	1.045
	" 19	3 p.m.	180	10		Refuses food.		1.025	1.022	1.025	1.022
							Sp. gr.				
II-A							H <sub>2</sub> O, per cent.				
							{ Whole blood. Serum. Cells.	79.3	82.2	82.6	85.4
								92.2	93.1	92.0	93.3
								66.1	66.1	67.4	66.2
							{ Serum. Cells.	153	148	146	135
								117	118	114	108
						Dog is fed during week following Experiment II-A.					
							{ Serum. Cells.	5.1	5.2	5.6	5.4
								8.2	8.7	8.3	10.0
							K " " 1000 "				

I-B	Jan. 28	11 a.m.	635	37	38	Refuses food.	Ca mm per 1000 gm. Serum.	2.9	2.1	3.7	2.9
	"	28	4.15 p.m.	120	7		Mg " " 1000 "	1.0	0.9	0.9	0.7
II-B	"	29	11 a.m.	120	7	29	Cl " " 1000 " {Whole blood Serum. Cells.	Lost. 102	88 110 56	88 109 55	89 104 54
							P(inorganic) mm per 1000 gm. Serum.	1.1	1.2	1.3	1.1

TABLE II.

*Experiment 2-H. Effect of Hemorrhage.*

ale; weigh 3.9 kilos. Calculated blood volume 1112 cc. Last fed Feb. 6; fasted throughout experiment. Given water daily.

Sample No.	Date.	Hour.	Volume of blood taken.	Per cent of total blood.	$\frac{\text{Weight of cells}}{\text{Weight of blood}} \times 100.$	Analytes.	Sample I.	Sample II.	Sample III.
I	Feb. 11	10 a.m.	425	38	47	Sp. gr.	1.057 1.024	1.049 1.021	1.040 1.021
						{ Whole blood. Serum.	80.2 92.5 66.3	82.2 93.9 67.6	86.2 93.7 66.8
						H <sub>2</sub> O, per cent.			
						CO <sub>2</sub> mm per 1000 cc.	18.9	21.0	17.7
						pH (electrometric).	7.34	7.32	7.35
II	" 11	2.30 p.m.	175	16	44	Protein (Kjeldahl), per cent.	6.22 33.97	5.13 32.67	5.26 32.41
						{ Cells.			
						Na mm per 1000 gm.	136 107	134 106	135 102

III	Feb. 12	10 a.m.	175	16	28	K mm per 1000 gm.	{Serum. Cells.	4.7 6.8	4.8 8.3	5.2 7.2
						Ca " " 1000 "	Serum.	2.7	2.7	2.3
						Cl " " 1000 "	{Whole blood. Serum. Cells.	84 109 56	85 107 57	89 100 61
						P(inorganic) mm per 1000 gm.	Serum.	1.0	1.5	Lost.



TABLE III.  
*Experiment 4-H. Effect of Repeated Hemorrhage.*

Dog 1, male; weight 21.4 kilos. Calculated blood volume, 1712 cc. Last fed June 7; fasted throughout experiment. Received water daily.

Sample No.	Date.	Hour.	Volume of blood taken. cc.	Per cent of total blood.	Weight of cells Weight of blood $\times 100$ .	Analyzes.	Sample I.	Sample II.
I	June 9	10 a.m.	550	32.4	52	Sp. gr.	1.059 1.025	1.042 1.020
	" 10	10 "	335	20		H <sub>2</sub> O, per cent.	79.1 93.0 66.5	85.0 93.3 65.8
	" 11	12 m.	425	25				
	" 12	10 a.m.	350	21	30	Na mm per 1000 gm.	145 102	136 102
II	" 13	11 "	450	26.5		K " " 1000 "	3.8 4.9	5.4 8.4
						Ca " " 1000 "	2.8	2.4
						Mg " " 1000 "	0.4	0.6

[illegible]

TABLE IV  
*Experiment 5-H. Effect of Repeated Hemorrhage.*

Dog 6, male; weight 22.0 kilos. Calculated blood volume 1760 cc. Received water daily.

Sample No.	Date.	Hour.	Volume of blood taken.	Per cent of total blood.	Weight of cells Weight of blood × 100.	Remarks.	Analyses.	Sample I.	Sample II.	Sample III.	Sample IV.	Sample V.
I	June 17	10-11 a.m.	750	43	56	Fasted 3 days before, and during ex- periment.	Sp. gr. { Whole blood. { Serum.	1.063 1.027	1.043 1.021	1.044 1.024	1.044 1.022	1.035 1.020
"	18	10 a.m.	400	23			H <sub>2</sub> O, per cent. { Whole blood. { Serum. { Cells.	76.6 91.5 64.8	85.4 92.8 65.6	84.2 92.3 65.4	84.3 92.3 66.9	87.8 93.7 68.4
"	19	10.30 a.m.	200	12	37	Gave 300 cc. water by stomach tube.	N a mm per 1000 gm. { Serum. { Cells.	141 104	118 102	131 93	126 96	138 105
"	19	4 p.m.	160	9		Gave 300 cc. H <sub>2</sub> O.						
"	20	10.30 a.m.	400	23	23							



TABLE V.

*Experiment 1-F. Effect of Fasting.*

Dog 7, weight 12.0 kilos. Calculated blood volume 960 cc. Fed once a day for a week previous to experiment; began fasting July 20. Received water daily.

Sample No.	Date.	Hour.	Volume of blood taken.	Per cent of total blood.	Weight of cells Weight of blood $\times 100$ .	Analyzes.	Sample I.	Sample II
I	July 20	10 a.m.	150	16	41	Sp. gr.  H <sub>2</sub> O, per cent.  Na mm per 1000 gm.	1.056 1.024	1.049 1.023
						{ Whole blood. { Serum.	81.0 91.4 66.2	82.8 93.1 67.0
						{ Whole blood. { Serum. { Cells.		
						{ Serum. { Cells.	139 108	136 109
II	" 27	10 "	200	21	39	K " " 1000 " Ca " " 1000 " Mg " " 1000 "	4.6 8.7	5.0 10.0
						Serum.	3.0	2.7
						"	0.9	0.9

[illegible]

TABLE VI.

*Experiment 2-F. Effect of Fasting.*

Dog 8, female; weight 16.6 kilos. Calculated blood volume 1328 cc. Fed once a day for a week previous to experiment; began fasting July 22. Received water daily.

Sample No.	Date.	Hour.	Volume of blood taken.	Per cent of total blood.	Weight of blood $\times 100$ .	Analyses.	Sample I.	Sample II.
I	July 22	10 a.m.	150	11	49	Sp. gr. H <sub>2</sub> O, per cent.	1.058 1.025	1.061 1.025
						{ Whole blood. Serum.	79.3	78.8
						{ Whole blood. Serum. Cells.	91.8 66.2	92.2 66.3
II	" 29	10 "	100	7	51	Na mm per 1000 gm.	135 113	134 114
						K " " 1000 "	5.1 9.0	5.0 8.9
						Ca " " 1000 "	3.0	3.1
						Mg " " 1000 "	0.9	1.2
						"		

[illegible]



In Experiment 5-H, on the 6th day, after 4 days of heavy blood losses and 1 day of recuperation, the changes observed were very similar to those of Experiment 4-H; *i.e.*, a fall of sodium and chloride in serum, a rise of potassium in cells. On the 6th day the dog was again bled heavily, and the next analysis made 2 days later. Although fasting, sodium and chloride increased sharply in the serum, but decreased in the corpuscles. Both potassium and inorganic phosphate increased remarkably in the cells, and inorganic phosphate increased in serum. The period of recovery was next studied in this dog, taking minimum blood samples (Nos. IV and V, Table IV) 3 days apart. In the 3 day recovery period without food, there were no changes of significance. The dog at this time was in good condition. After 3 more days with food, the serum had returned to its original normal composition, except for proteins. In the cells, potassium and inorganic phosphate remained at a high level, while sodium became normal.

The determinations of inorganic phosphates in the cells of Experiments 1-H-A and B and 2-H were discarded because they were high and variable, presumably due to overheating while centrifuging. Buell (1923) found no inorganic phosphate in corpuscles, and believes the commonly found inorganic phosphate in corpuscles is due to hydrolysis of organic phosphorus compounds. In experiments 4-H and 5-H, however, the time of centrifuging was exactly  $\frac{1}{2}$  hour, so that the increase in inorganic phosphate in Experiment 5-H is thought to be of significance.

The control Experiments 1-F and 2-F were undertaken to determine whether or not 1 week's fasting alone might cause changes in the inorganic composition of blood. Tables V and VI show practically no change in any of the ions in either serum or corpuscles.

In spite of all the care taken in the determination of chlorides in whole blood and serum, the calibration of the pipettes against each serum and blood, and the careful measurement of weight hematocrit, we are not satisfied with the calculated concentration of chlorides in cells. The variations in the chloride ratio between serum and cells are not those expected from the changes in pH known to occur after hemorrhage. In two cases the ratios are greater than unity, which is obviously incorrect, according to our knowledge of the distribution of chlorides governed by the Donnan equilibrium.

For the purpose of studying the osmotic relationships, all the data of the accompanying tables were recalculated as millimols per kilo of water, but as no new information was gained, the tables were omitted.

#### DISCUSSION.

After severe hemorrhage the organism employs every possible means to secure normal nutrition of the tissues by restoring blood volume. The dependence of a normal blood flow upon the restoration of blood volume has been shown by Gesell, Capp, and Foote (1922-23). That the inflow of tissue fluids is an important factor in restoring blood volume after hemorrhage has long been recognized. In an investigation of the changes which take place in the chemical composition of the blood after hemorrhage, it becomes necessary therefore to learn something of the nature of the tissue fluids. Furthermore such an investigation naturally involves the factors which control the normal composition and volume of plasma. Among these may be mentioned the activity of the kidney in governing to a great extent plasma volume as well as its content of salts. Other factors of great importance are the permeability of the capillaries, and the passage of fluids and ions through their walls. Changes in blood pressure, osmotic pressure, and in the protein concentration of the plasma and lymph may influence these factors in various ways.

It is important to know the effects of hemorrhage on these various mechanisms. It has been observed by various investigators that hemorrhage may reduce blood pressure sufficiently to stop or retard the excretion of urine. When the blood pressure is raised by inflow of fluids from the tissues or gastrointestinal tract, urine excretion again begins. The cessation of urine flow after severe hemorrhage therefore aids the organism in restoring blood volume by conserving both water and salts, but limits its ability to regulate the concentration of the individual ions.

The passage of fluids and ions through capillary walls is an important mechanism for regulating plasma volume and composition, as already stated. That tissue fluids actually pass into the blood stream after severe hemorrhage and consequent lowering of blood pressure is unquestioned, although the mechanism by which it occurs is not well understood (Krogh, 1922). Al-

though there may be delay in the dilution of the blood, if the animal lives blood volume eventually rises due to inflow of these fluids. Carrier, Lee, and Whipple (1922) found that after hemorrhage the dilution of the blood is complete in 18 to 24 hours. Robertson and Bock (1919) on the other hand believe that without forced ingestion of water, blood volume may remain abnormally low for days. They observed that the organism retains great quantities of ingested water after hemorrhage until blood volume is fully restored. Lee, Carrier, and Whipple (1922) state that copious water ingestion may even raise the plasma level of a normal animal 10 to 40 per cent above normal. The fact that water given by mouth or rectum may aid in the restoration of blood volume indicates the necessity of considering the absorption of fluids from the gastrointestinal tract as well as the inflow of tissue fluids in the dilution of the blood after hemorrhage. It seems likely that the response of the body to hemorrhage in restoring blood volume may differ widely according to the availability of fluids from the various sources. In the absence of excess water in the gastrointestinal tract it is evident that the initial dilution of the blood must be caused by inflow of the extracellular fluid or lymph, which is in a state of constantly changing equilibrium with plasma on the one hand and with the intracellular fluids on the other. As blood volume is restored, the extent to which intracellular fluids are drawn into the blood depends upon the quantity of lymph which can be drained from the tissue spaces, and upon the extent to which the intracellular fluids compensate for the loss of lymph. An abundant water supply in the gastrointestinal tract should certainly have a sparing action on the use of the intracellular fluids.

That intracellular fluids are actually drawn upon is shown by Gamble, Ross, and Tisdall (1923) in their study of the metabolism of fixed base during fasting. They found that in two cases of fasting children, five to six times as much intracellular as extracellular body water was lost. They furnished evidence that the destruction of cellular protoplasm resulted in the passage of a proportionate amount of the cellular water and salts into the blood, and thence into the urine. These facts lead us to expect that a considerable proportion of the tissue fluids entering the blood after hemorrhage should be intracellular fluids with their salts. That

cellular protoplasm is actually destroyed is indicated by the increased nitrogen metabolism occurring after hemorrhage. Hawk and Gies (1904) found an increased excretion of nitrogen in the urine, Taylor and Lewis (1915) found an increase in the urea and non-protein nitrogen of the blood, and Gy  rgy and Zunz (1915) noted an increase in the amino acids of the blood. That there is great destruction of tissue after hemorrhage is also evident from the loss of weight.

If lymph, intracellular fluids, and fluid absorbed from the gastrointestinal tract differ appreciably from each other and from plasma in composition, it should be possible by investigating what changes occur in the blood after hemorrhage to determine the nature of the diluting fluids and thus their origin. Such an investigation becomes of interest in view of the hypothesis of Milroy (1917) that the fluids entering the blood after hemorrhage are hypotonic. It was hoped that this research would throw light on this point, and would aid in determining whether or not the intracellular fluids with their peculiar inorganic composition enter the blood after hemorrhage, and change its composition.

It is evident, therefore, that a knowledge of the composition of both extracellular and intracellular fluids is necessary. The literature on this subject is very meager. The only analyses of dog lymph found were those of Petersen and Hughes (1925), but their figures are so variable that the analyses of human lymph by Munk and Rosenstein (1891) are given. The inorganic composition of human, horse, and dog lymph appears to be very similar, judging from the few analyses available. The analyses of dog muscle are those of Katz (1896). The average of the analyses of the normal serum and corpuscles of the dogs studied in this and the following paper are given (see Table VII).

The data of this table give us a basis for discussing the changes found in the blood after hemorrhage. It is evident that if the dilution of the blood is accomplished only by absorption of water from the gastrointestinal tract, there should result a decrease in all the inorganic constituents and protein of the serum, assuming that this liquid would have a low salt content. Dilution by lymph only should result in a decrease in serum potassium and possibly protein. Intracellular fluid only should produce a decrease in sodium and chloride, and an increased concentration of potas-

sium and magnesium. It is obvious that both lymph and fluid from the gastrointestinal tract aid in restoring blood volume, and the question is merely whether intracellular fluids are actually drawn upon. The results of dilution of the blood by intracellular fluids combined with either one of the other two fluid sources are self-evident. We actually find on analysis of serum a slight fall of sodium after a single hemorrhage, and a greater decrease after repeated hemorrhage. Potassium, magnesium, and calcium however remain unchanged. A normal potassium level after hemorrhage can only be accounted for by a partial dilution with intracellular fluids. That sodium and chlorides decrease is confirmatory evidence. The inflow of intracellular fluids in the proportion that may be assumed from the work of Gamble, Ross, and Tisdall (1923) should however cause an increased concentration

TABLE VII.

*Comparison of the Inorganic Composition of Muscle, Lymph, Serum, and Corpuscles.*

Substance.	H <sub>2</sub> O	Na	K	Ca	Mg	Cl
	per cent	mm per 1000 gm.	mm per 1000 gm.	mm per 1000 gm.	mm per 1000 gm.	mm per 1000 gm.
Dog muscle.....	76.4	41	83.4	1.75	9.87	22.8
Human lymph.....		140	3.17	2.72	1.03	101
Dog serum.....	92.5	141	5.19	2.90	0.82	106
" corpuscles.....	66.1	109	7.77			55.8

both of potassium and of magnesium that could not escape detection, were it not for the fact that the excess of these cations may be excreted by the kidney or stored by some other tissue. For this reason it is significant that the potassium concentration in the corpuscles increases remarkably after repeated hemorrhage. The total evidence indicates that a considerable part of the dilution of the blood is due to intracellular fluids.

The most surprising change in blood composition after hemorrhage is however not in serum, but in the corpuscles. In each of the experiments recorded in the accompanying tables there were observed changes in the potassium content of the corpuscles. The changes were small after a single hemorrhage, and large after repeated hemorrhage. In Experiments 1-H-B, 4-H, and 5-H the

changes were far beyond the limits of experimental error of the determinations, and were too great to be accounted for by changes in the water content of the corpuscles. In Experiment 5-H, for example, the potassium concentration rose from 6.5 mM to 15.2 mM per 1000 gm. of cells, while the water content actually increased from 64.8 to 65.4 per cent.

These changes point to a possible permeation of the corpuscle wall by potassium ions. Frequent references are found in the literature to the impermeability of the red blood corpuscle to cations. It has been shown by Doisy and Eaton (1921) that variations in  $\text{CO}_2$  do not cause a shift of sodium and potassium between plasma and corpuscles, which earlier workers believed took place. Ashby (1924) found red blood corpuscles permeable to potassium in aqueous salt solutions. Their permeability to sodium was much less than to potassium.

The variations of sodium in the corpuscles are also interesting. In Experiments 1-H-B and 2-H the fall of sodium is only a little beyond the experimental error. In Experiment 4-H there is no change, but in Experiment 5-H sodium decreased in the corpuscles far beyond the limits of error. In this experiment the low sodium in the corpuscles parallels the lowered concentration of sodium in the serum, and suggests a loss of sodium from the corpuscles.

In order to determine whether ions pass in and out of corpuscles it is necessary to recalculate all of the results, correcting for water shifts. These calculations were made, and as the observed water shifts were small, the relationships were not changed. Therefore the data are not presented although they may be calculated from the tables.

It has already been pointed out that repeated hemorrhage causes a passage of intracellular tissue fluids, potassium-rich and sodium-poor, into the blood stream. Although the lowered level of sodium is shared by both corpuscles and serum, at least in Experiment 5-H, increases in cell potassium are not paralleled by any changes of potassium in the serum. The corpuscles seem to be exhibiting a selective action for potassium although there is no information available to suggest why there should be increased selective action after hemorrhage. The selective action of tissues in general for potassium is seen in the high potassium content of muscle, and in the fact that corpuscles contain more potassium than the serum in which they are suspended.

In searching for an explanation of the high cell potassium, the possibility of the formation of new corpuscles rich in potassium must be considered. A single hemorrhage will cause a sudden washing into the blood stream of the reserves of red cells in the bone marrow. This does not influence the average corpuscle composition as observed 24 hours after hemorrhage. The reserves therefore appear to be no higher in potassium concentration than the cells already in the blood stream. Repeated hemorrhage should produce the maximum stimulus to rapid formation of new red cells, and it is after repeated hemorrhage that the rise in cell potassium is observed. At this time the body has been depleted of its sodium, while excessive quantities of potassium are being set free by destruction of cellular protoplasm. It is thus possible that the organism makes use of the more available potassium in forming new corpuscles. The high potassium values in corpuscles after repeated hemorrhage may possibly be associated with the disturbances which occur in serum and cell proteins. Foster and Whipple (1921-22) have shown that the concentration of fibrinogen in plasma may fluctuate widely after hemorrhage, often greatly exceeding its normal concentration. Inagaki (1907) noted marked changes in the albumin-globulin ratio in the serum of rabbits on the days following severe hemorrhage. The proportion of globulin to albumin was greatly increased, although the total serum proteins were diminished. Inagaki also observed that after hemorrhage the red corpuscles became smaller, and lost a protein which he believed was globin. Scott (1915-16) confirmed this observation by finding that a protein passed from corpuscles to serum when blood was diluted with Ringer's solution. Serum proteins were determined in Experiments 2-H, 4-H, and 5-H, and were found to decrease after hemorrhage in each experiment. In Experiment 2-H the total nitrogen of corpuscles was determined by the Kjeldahl method, and a definite decrease was found, confirming the observations of Inagaki and Scott. It is difficult to associate a loss of protein from the cells with a gain in potassium.

In a discussion of variations of the inorganic constituents of blood it would seem that the theories of Milroy (1917) and of Henderson and Haggard (1922) should be mentioned. Milroy observed after hemorrhage a diminished specific gravity of whole blood, and a diminished alkaline reserve of plasma, which was

slightly hemolyzed. He concluded that hypotonic tissue fluids low in bicarbonate entered the blood after hemorrhage. The lowered specific gravity of whole blood was undoubtedly mainly due to diminished concentration of cells. The diminished cation and protein concentrations found in the present work indicate that the osmotic pressure of the blood is probably lowered after hemorrhage, though no hemolysis was ever observed. Henderson and Haggard (1922) propose the hypothesis that the low alkaline reserve after hemorrhage is due to diffusion of base from the blood into the tissues. The changes in base necessary to account for maximum changes in alkaline reserve are so small compared with the total base of blood that the variations might be within the errors of our experiments. But considering the low alkaline reserve immediately after hemorrhage and the high alkaline reserve shown to occur later by Evans (1921) and Bennett (unpublished), the fact that sodium and chloride rise and fall in equivalent quantities suggests that the diffusion of base as a bicarbonate salt in and out of the blood does not account for the variations.

#### SUMMARY.

1. The effects of hemorrhage on the inorganic composition of serum and corpuscles have been studied.

2. Some modifications of existing methods of analysis for the inorganic constituents of serum and corpuscles are described.

3. A single hemorrhage, amounting to from one-third to one-half of the total blood volume results in a slight decrease in both sodium and chloride of serum, and no significant change in corpuscles.

4. Repeated severe hemorrhage produces a greater fall of sodium and chloride of serum, and an increase in the inorganic phosphate. No changes in the potassium, calcium, or magnesium of serum were observed. A large increase in the potassium concentration of the corpuscles occurred.

5. Possible causes for the high cell potassium are discussed, but the experimental data are insufficient to warrant any conclusions.

The writer desires to express to Dr. D. Wright Wilson, who suggested this study, his sincere thanks for many valuable suggestions, and much helpful criticism in both this and the following paper.



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# STUDIES ON THE INORGANIC COMPOSITION OF BLOOD.

## II. CHANGES IN THE POTASSIUM CONTENT OF ERYTHROCYTES UNDER CERTAIN EXPERIMENTAL CONDITIONS.

By STANLEY E. KERR.

*(From the Department of Physiological Chemistry, University of Pennsylvania, Philadelphia.)*

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### INTRODUCTION.

The red corpuscle has been generally regarded as impermeable to the cations sodium, potassium, calcium, and magnesium. In the preceding paper (Kerr, 1926), however, it has been shown that the concentration of potassium in the red cells of the dog increases after repeated hemorrhage. The fact that no satisfactory explanation for this change could be found suggested a further study of the variations of potassium in blood corpuscles. It was proposed therefore to investigate first whether conditions other than repeated hemorrhage might cause changes in the concentration of potassium in the corpuscles. A comparison of the conditions causing the changes should determine whether increases in concentration are due to a storage of potassium in a non-diffusible form, or whether potassium diffuses freely through the corpuscle membrane, shifting in and out as certain investigators in the past believed.

Hamburger (1916) believed that there was a transfer of base between serum and corpuscles due to changes in  $\text{CO}_2$  tension. J. Mellanby and Wood (1923), and Collip (1921), came to the same conclusion from their analyses of ashed serum. Doisy and Eaton (1921), however, showed that these shifts of base were not real, but appeared because of the failure of previous investigators to correct for the water shift between serum and corpuscles. Van Slyke, Wu, and McLean (1923) in their presentation of the relationships between corpuscles and serum have assumed that the corpuscles are impermeable to sodium and potassium.

A disturbance of the normal value for potassium in the serum would appear to be the most favorable condition for producing a similar change in the corpuscles. The obvious method for accomplishing this would seem to be to inject a potassium salt into the blood stream and subsequently examine the corpuscles for an increase in potassium. Whelan (1925) found no change in the cells under these conditions. As a trial experiment to test her findings, serum containing an added excess of potassium chloride was added to corpuscles in a test-tube. After 1 hour the corpuscles were separated and analyzed. No change in potassium was found.

In order to study the effect of lowering or raising the level of potassium in the plasma on the corpuscle composition, three groups of experiments were undertaken.

1. Gelatin-saline solution was injected intravenously into dogs in order to lower the plasma potassium by dilution of the blood. The gelatin was added to prevent loss of the injected fluid through the capillary walls (Bayliss, 1915-17).

2. The potassium concentration of plasma was lowered by overdosage of insulin. This effect of insulin was observed by Briggs, Koechig, Doisy, and Weber (1923-24) and by Harrop and Benedict (1924).

3. Dogs were given subcutaneous injections of sodium oxalate. Gross (1923) had found that injection of oxalate caused the concentration of potassium in whole blood to rise to an abnormally high level. Since he did not determine whether this change occurred in serum or in corpuscles, it seemed important to determine the potassium distribution.

### *Methods.*

All of the experiments were conducted on dogs. [Blood for analysis was studied throughout in the manner described in the preceding paper (Kerr, 1926). The analytical methods likewise were the same as previously used. Experience gained in the determination of potassium and sodium made certain the detection of small changes in concentration. Glucose was determined by the method of Folin and Wu (1919), inorganic and hydrolyzable phosphorus by the method of Briggs (1924).

## EXPERIMENTAL.

*Effect of Diluting the Blood with Gelatin-Saline Solution.*

In order to avoid the plethora which would result from the injection of a large quantity of diluting fluid into the blood, the injection was in each case preceded by the withdrawal from the heart of a volume of blood equal to the amount of fluid to be injected. Three experiments of this kind were conducted (Experiments 1-G, 2-G, and 3-G, Tables I, II, and III), on fasting dogs.

In the first experiment (Table I), approximately half the blood of the dog was removed rapidly from the heart and jugular vein. Immediately after the withdrawal of the blood an equal volume of 0.95 per cent sodium chloride solution containing 6 per cent gelatin was injected slowly into the jugular vein, the injection requiring about half an hour. Samples were drawn from the heart for analysis 5 minutes after the completion of the injection to study the immediate effects, again  $5\frac{1}{2}$  hours later, and finally 24 hours after the injection of gelatin. 5 minutes after the injection, the effect of addition of the saline is evident in the increased serum sodium and chloride, and the lowered potassium, the latter being merely a result of dilution. The absence of a drop in calcium is due to the fact that the gelatin, as shown by analysis, contained calcium. In the corpuscles there was a slight increase in potassium. After  $5\frac{1}{2}$  hours sodium, chloride, and potassium have returned to normal levels in serum, and remained so at the 24 hour period. There were no changes in the corpuscles at the  $5\frac{1}{2}$  hour period, but 24 hours after the injection there was a great increase in the cell potassium, the concentration more than doubling.

The interpretation of this change in potassium is obscured by two facts—the presence of the gelatin and the three losses of blood when large samples were taken for analysis. The original purpose of the experiment—to determine whether a dilution of the serum potassium would cause a fall in cell potassium—was accomplished in that no fall was observed. The subsequent rise, interpreted in the light of the high cell potassium found after repeated hemorrhage, might be due either to the presence of the gelatin, or to the repeated hemorrhage.

A second experiment (Table II) was undertaken in which the initial hemorrhage was purposely made a large one, about 40

TABLE I.  
*Experiment 1-G. Effect of Hemorrhage, Followed by Intravenous Injection of Gelatin-Saline Solution (6 Per Cent Gelatin in 0.95 Per Cent NaCl).*

Dog 3, female; weight 11.4 kilos. Calculated blood volume 912 cc.

Sample No.	Date.	Hour.	Volume of blood taken.	Per cent of total blood.	Weight of cells $\times 100$ .	Remarks.	Analyses.	Sample I.	Sample II.	Sample III.	Sample IV.
I	Feb. 21	10 a.m.	200 cc.	22	43	Normal blood sample after 2 day fast. Fed after this sample, but fasted through remainder of experiment.	Sp. gr. { Whole blood. Serum.	1.053 1.022	1.030 1.021	1.031 1.019	1.030 1.020
							H <sub>2</sub> O, per cent. { Whole blood. Serum. Cells.	81.7 93.6 66.1	91.2 94.6 66.5	90.5 95.2 66.0	90.1 94.5 66.5
	"	23 10.45 a.m.	440	48		Hemorrhage.	pH (electrometric). Serum.	7.3	7.3	7.4	7.3
	"	23 10.45-11.30 a.m.				Gelatin-saline solution (440 cc.) injected.	CO <sub>2</sub> mm per liter. "	19.6	13.8	12.2	14.6
II	"	23 11.35 a.m.	200	22	12		Na " " 1000 gm. { Serum. Cells.	136 111	144 109	136 110	138 108

III	Feb. 23	5 p.m.				K mm per 1000 gm.	{Serum. Cells.	5.8	3.8	5.0	5.3
								7.1	8.5	8.1	17.7
						Ca " " 1000 "	Serum.	2.8	3.0	2.5	2.0
						Mg " " 1000 "	"	0.7	0.6	0.6	0.6
IV	" 24	11 a.m.				Cl " " 1000 "	{Whole blood. Serum. Cells.	87	108	104	100
								112	118	112	107
								56	33	61	58
								Found dead.			
								200	22	16	
								200	22	16	
								200	22	16	
								200	22	16	

TABLE II.  
*Experiment 2-G. Effect of Hemorrhage, Followed by Intravenous Injection of Gelatin-Saline Solution (3 Per Cent Gelatin in 0.80 Per Cent NaCl Solution).*

Dog 9, male; weight 6.4 kilos. Calculated blood volume 512 cc. Received water daily.

Date.	Hour.	Volume of blood taken.	Per cent of total blood.	Volume of cells Volume of blood X 100.	Remarks	Serum.		Corpuscles.	
						H <sub>2</sub> O	K	H <sub>2</sub> O	K
		cc.				per cent	mm per 1000 gm.	per cent	mm per 1000 gm.
Aug. 10	11 a.m.	200	39	33	Last fed Aug. 8; fasted throughout experiment.	93.2	4.7	66.2	7.3
" 10	11.15-11.45 a.m.				Injected 200 cc. gelatin-saline into jugular vein.				
" 11	11.30 a.m.	80	16	25		93.7	3.9	66.6	9.3

TABLE III.  
*Experiment 3-G. Effect of Hemorrhage, Followed by Intravenous Injection of Gelatin-Saline Solution (9 Per Cent Gelatin in 0.85 Per Cent NaCl Solution.)*

Dog. 10, female; weight 8.8 kilos. Calculated blood volume 704 cc. Received water daily.

Date.	Hour.	Volume of blood taken.	Per cent of total blood.	Volume of cells Volume of blood × 100.	Remarks.	Serum.		Corpuscles.	
						H <sub>2</sub> O	K	H <sub>2</sub> O	K
		cc.				per cent	mm per 1000 gm.	per cent	mm per 1000 gm.
Aug. 24	10.30 a.m.	200	28	42	Last fed Aug. 20; fasted throughout experiment.	92.5	5.0	66.5	8.2
" 24	11-11.30 a.m.				Injected 175 cc. gelatin-saline into jugular vein.	93.5	5.9	66.4	8.3
" 25	10.30 a.m.	100	14	28					



per cent of the total blood volume, the blood thus lost being replaced by an equal volume of 3 per cent gelatin in 0.80 per cent sodium chloride. The next blood sample was taken 24 hours later, so that changes could not be ascribed to repeated hemorrhages. Examination of Table II shows that the potassium concentration of the corpuscles increased considerably, while the potassium in serum fell. The fall in hematocrit reading equaled that calculated from the blood lost, indicating no regeneration of red cells. Since single hemorrhages without gelatin injection produced no such effects, the rise in potassium would seem to be due to the gelatin.

A third experiment (Table III) was conducted with the purpose of introducing a large quantity of gelatin into the blood stream with as small a withdrawal of blood as possible. After a hemorrhage amounting to 28 per cent of the total blood volume, a 9 per cent solution of gelatin in 0.85 per cent sodium chloride was introduced intravenously, giving a concentration of about 2.3 per cent gelatin in the blood stream. 24 hours later there was found a small rise in the potassium concentration of the serum but no change in the cells.

That gelatin causes changes in the blood can hardly be doubted, for the arterial blood even 5 minutes after the injection was of a dark color, and the corpuscles settled with great rapidity after defibrination. Hanzlik, De Eds, and Tainter (1923) have also reported this change in color and sedimentation after gelatin and gum acacia injections.

These experiments enable us to conclude only that heavy bleeding followed by injections of gelatin-saline cause the potassium concentration of corpuscles to increase. It remains uncertain whether the change in serum protein accounts for this altered distribution of potassium.

#### *Effect of Insulin.*

In order to produce a lowered serum potassium and to study its effect on the concentration of potassium in the corpuscle, dogs were given large doses of insulin. Briggs, Koechig, Doisy, and Weber (1923-24) observed that large doses of insulin caused a definite decrease in serum potassium and inorganic phosphate before the onset of convulsions. Their few analyses on whole blood led them

TABLE IV.  
*Experiment 1-Ins. Effect of Insulin.*  
 Dog 10, female; weight 8.8 kilos. Calculated blood volume 704 cc.

Date	Hour.	Volume of blood taken.	Volume of cells × 100.	Insulin injected, clinical units.	Remarks	Serum.					Corpuscles.		
						H <sub>2</sub> O	Glucose.	Inorganic P.	K	Protein.	H <sub>2</sub> O	K	Inorganic + hydrolyzable P.
		cc.				per cent	mg. per 100 gm.	mm per 1000 gm.	mm per 1000 gm.	per cent	per cent	mm per 1000 gm.	mm per 1000 gm.
Aug. 13	10.25 a.m.	70	43	20	Last fed 20 hrs. before experiment.	92.7	120	1.3	5.2	6.1	66.6	7.4	4.5
" 13	10.35 "			10	Normal blood sample.								
" 13	11.35 "			10									
" 13	12.20 p.m.			10									
" 13	1.15 "	70	40		Dog depressed but no signs of hyperirritability.	93.4	40	0.55	3.5	5.3	66.1	8.4	4.0

TABLE V.  
*Experiment 2-Ins. Effect of Insulin.*  
 Dog 11, male; weight 7.3 kilos. Calculated blood volume 584 cc.

Date.	Hour.	Volume of blood taken.	Volume of cells × 100.	Insulin injected, clinical units.	Remarks	Serum					Corpuscles.	
						H <sub>2</sub> O	Glucose.	Inorganic P.	K	Protein.	H <sub>2</sub> O	K
						per cent	mg. per 100 gm.	mm per 1000 gm.	mm per 1000 gm.	per cent	per cent	mm per 1000 gm.
Aug. 17	10.15 a.m.	60	41		Last fed Aug. 14. Normal blood sample.	92.6	143	1.9	3.9	6.1	66.8	9.6
"	10.20 "			40								
"	12.30 p.m.			30								
"	1.50 "			30								
"	3.00 "				Dog begins to appear drowsy.							
"	3.05 "	50	35			93.4	46	1.1	2.8	5.3	67.5	10.3



to state that there seemed to be a loss of potassium from the corpuscles as well as from serum. Harrop and Benedict (1924), studying the effect of insulin upon human diabetics and on normal rabbits, found that potassium, inorganic phosphates, and glucose in serum decreased after insulin, the potassium dropping to as much as half of its original level. Blood samples were taken before the injections, and again 2 or 3 hours afterwards, during the period of depression preceding the onset of hyperirritability. The details of procedure and the analytical results are presented in Tables IV, V, and VI.

In spite of the large doses of insulin injected, the first two dogs showed no signs of hyperirritability, although they were uneasy and depressed. The third dog went into convulsions within less than a minute after the injection. This could not have resulted so quickly from the hormone injection.

In the first two experiments, the fall in serum glucose and in inorganic phosphate was accompanied by a marked decrease in serum potassium. In both cases there was a simultaneous *rise* in the potassium content of the corpuscles. In the third experiment, which was complicated by convulsions from an unknown cause, there was no change in serum potassium or phosphate, and glucose did not fall to a low level. In the corpuscles, however, there was a great increase in potassium concentration. In the insulin experiments the increased concentration of potassium in the corpuscles was usually accompanied by an increase in the water content of the corpuscles. Correction for this water shift shows that the transfer of potassium into the cells is even greater than the table indicates. In each of the three experiments there was a fall in serum proteins after the insulin injections. This may have been due to withdrawal of the fairly large blood samples immediately before the insulin injections, since the amount taken was nearly 10 per cent of the total blood volume. It is quite possible, however, that the fall in serum proteins was a direct result of the insulin, in which case it may be of significance in connection with the changed potassium distribution.

#### *Effect of Oxalate Poisoning.*

Gross (1923) studied the effects of injections of sodium oxalate on the inorganic constituents of the whole blood of dogs. He

TABLE VII.  
*Effect of Sodium Oxalate Injections.*

Dog 8, female; weight 15.3 kilos. Fasted 1 week before experiment. Received water daily. Calculated blood volume, 1224 cc.

Sam- ple No.	Date.	Hour.	Vol- ume of blood taken.	Weight of cells Weight of blood × 100.	Remarks.	Analyses.	Sample I.	Sample II.
I	July 29	10 a.m.	100	51	Fasted July 22-31. Blood sample.	H <sub>2</sub> O, per cent.	78.8 92.2 66.3	81.4 93.4 65.2
	" 29	4 p.m.			Injected 1 gm. so- dium oxalate in 30 cc. H <sub>2</sub> O.	{Serum. Cells.		
	" 30	11 a.m.			Injected 1 gm. so- dium oxalate in 30 cc. H <sub>2</sub> O.	{Serum. Cells.	135 114	135 115
	" 30	4.30 p.m.			Injected 0.66 gm. sodium oxalate in 20 cc. H <sub>2</sub> O. No signs of tetany.	{Serum. Cells.	5.0 8.9	9.7 11.3
	" 31	10 a.m.			Injected 1 gm. so- dium oxalate in 30 cc. H <sub>2</sub> O. No tetany.	{Serum. Cells.	3.1 1.3	2.2 1.2
	" 31	1 p.m.	125	42	Blood sample.	{Whole blood. Serum. Cells.	76 105 49	62 88 27
II	" 31	3 "			Died. No tetany.	P (inorganic) mm per 1000 gm.	1.1 1.9	4.3 1.7
	" 31	3 "				Protein, per cent.	6.7	5.6

found a great increase in potassium and inorganic phosphate, with a decrease in calcium, sodium, and chloride. The distribution of these elements between corpuscles and serum was not investigated.

A single experiment was therefore carried out to determine whether the changes noted by Gross involved changes in the corpuscles as well as in the serum. The results are given in Table VII. Although the dosage of oxalate injected was about the maximum used by Gross, the dog did not develop tetany, serum calcium falling only to 2.2 mm per 1000 gm. Sodium remained unchanged in both serum and cells, while chlorides fell to levels much lower than normal. The potassium concentration in serum almost doubled, and in corpuscles there was an unmistakable rise. The rise in cell potassium appears to be smaller than in serum, but when the concentrations are stated in terms of millimols per kilo of water it is seen that the cells have shared almost equally in the increase. The decrease in water content of the corpuscles accounts for only 0.2 mm of the increase in potassium. Inorganic phosphates in serum increased remarkably. Serum proteins decreased to a considerable extent. The changes most significant for the purposes of this paper are the great increases of both potassium and inorganic phosphate in the serum, and of potassium in the cells. The change in serum proteins may be of importance in influencing the new potassium distribution.

#### DISCUSSION.

In each of these three groups of experiments, the changes in the concentration of potassium in the corpuscles are of such a nature that there can be little doubt that potassium actually passes from the plasma into the cells. After insulin injections the changes are so rapid that the potassium concentration in the corpuscles rises within 2 hours. Doisy and Eaton (1921) have shown that potassium does not shift in and out of corpuscles due to changes in carbon dioxide tension. As the change after oxalate injection involves an equimolar increase in both cell water and serum water, the obvious inference is that free diffusion may have occurred, but it should be noted that both before and after injection the concentration of potassium in the cell water is higher than in serum water, so that some other explanation must be considered. Since the

potassium concentration may increase in the cells while it increases, decreases, or remains unchanged in the serum, the conclusion seems inevitable that potassium may under some conditions enter the corpuscle readily and be stored in a non-diffusible form. Changes in serum proteins and inorganic phosphates may however be of significance.

#### SUMMARY AND CONCLUSIONS.

1. Changes in the potassium concentration of serum and corpuscles were studied.
2. A single large hemorrhage followed by gelatin-saline infusion caused the potassium concentration of the corpuscles to increase.
3. Overdosage with insulin resulted in a fall of serum potassium and a rise of cell potassium.
4. Subcutaneous injections of sodium oxalate caused a large increase in potassium of both serum and corpuscles.
5. The changes in potassium concentration of the corpuscles cannot be accounted for by shifts of water, but only by an actual penetration into the cell by potassium.
6. It would appear that, though the red corpuscle is permeable to potassium, it must be combined to some extent in non-diffusible form.

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## CLINICAL CALORIMETRY.

### XLI. THE STORAGE OF GLYCOGEN IN EXOPHTHALMIC GOITER.

By HENRY B. RICHARDSON, SAMUEL Z. LEVINE, AND EUGENE F. DU BOIS.

WITH THE TECHNICAL ASSISTANCE OF G. F. SODERSTROM.

(*From the Russell Sage Institute of Pathology in Affiliation with the Second Medical (Cornell) Division of Bellevue Hospital, New York.*)

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#### INTRODUCTION.

The object of the work to be described in this paper was to study the storage of carbohydrate in exophthalmic goiter. Observations have been reported which appear to support the theory that there is in this disease an impairment in the mechanism by which glycogen is stored. In 1922, Sanger and Hun (1) tested the effect of the ingestion of glucose, and found that the blood sugar curve had a higher and broader peak than in normal individuals, whereas the respiratory quotient, as Du Bois had shown in 1916 (2), gave no evidence of any defect in the oxidation of carbohydrate. Sanger and Hun explained the results as due to a deficiency in the ability to store carbohydrate, and cite in support of this conclusion the work of Cramer and Krause (3), Kuriyama (4), and Parhon (5), who found that feeding with thyroid reduced the hepatic glycogen, even if the diet contained an ample supply of carbohydrate. Evidence as to the validity of this theory is to be presented below.

#### *Method.*

#### *Estimation of Glycogen.*

It is impossible, of course, to determine chemically the quantity of glycogen present at any one time in the human organism. If, however, the source of supply is cut off and the amount used determined over a sufficiently long period, an estimate may be made.

Glycogen is converted to glucose, which normally is oxidized to carbon dioxide and water, and the quantity thus destroyed may be measured by the respiratory exchange in conjunction with the nitrogen of the urine. Theoretically speaking, the determinations should be continued over a period sufficiently long to ensure the removal of the entire store of glycogen, and the conditions should be such as to attain this end. Judging from experiments on animals, this would require shivering, adrenalin, and even phlorhizin or strychnine. In man, therefore, it is practicable to remove only that portion of the glycogen which can be oxidized under ordinary conditions. This being the case, it is more important to maintain conditions which are strictly comparable as between different subjects, than to attempt to remove all the glycogen. We made observations on resting patients which had the further advantage that the fasting subject L. studied by F. G. Benedict (6) could be used for a control. The assumption under which we worked was that the quantity of glycogen which escaped measurement at rest was the same in the different individuals, whether thyrotoxic or normal.

When the intake of carbohydrate ceases, the supply of glycogen is cut off, with the possible exception of that arising from endogenous protein. If the latter were converted into glycogen and then oxidized, the results would be the same as if the oxidation were direct, a process for which allowance is made in the calculations. If, however, a portion were laid down as glycogen an error would be introduced. For example, if the whole of the glucose which is derived from protein were stored in the body the effect would be the same as if it were excreted, and the calculations used by Lusk for diabetes of maximum severity, as described in the paper by Allen and Du Bois (7), would apply. For a given respiratory exchange this method would give a higher non-protein quotient than the ordinary computation, likewise a larger amount of carbohydrate oxidized, and a greater quantity of glycogen in storage. The estimate arrived at by the usual calculation would be subject to a plus correction. If less than the total quantity of glucose derived from protein were stored, the error would be smaller but in the same direction. No method is at hand for determining the size of this correction, which is therefore assumed to be the same in different subjects.

The possibility of the formation of glycogen from glycerol may be mentioned, although it would introduce no appreciable error into the calculations. Shaffer (8) has constructed a table<sup>1</sup> from which may be calculated the quantity of carbohydrate oxidized, supposing all of the glycerol of the fat to be converted into glucose, and only the fatty acid oxidized. In the case of Henry D. the necessary data are the oxygen consumed, as shown in Column 4 of Table II, and the non-protein respiratory quotients, which were

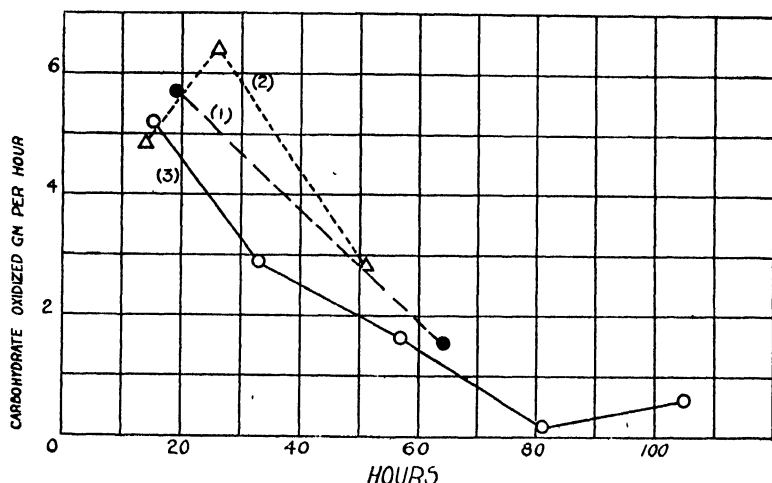


FIG. 1. The storage of glycogen in exophthalmic goiter. The curves show the oxidation of carbohydrate. Curves 1 and 2 are from patients; Curve 3 from a normal individual. The relative quantities of glycogen in storage are shown by the areas under the respective curves.

0.773, 0.766, and 0.739 respectively. Using Shaffer's table, it is found that 0.17, 0.30, and 0.47 gm. of carbohydrate would have been metabolized in excess of the amounts calculated by the ordinary method. Plotting this in the form of a curve, as in Fig. 1, and measuring the area beneath gives 13 gm. as the amount to be added to the estimate of glycogen in storage. This is the maxi-

<sup>1</sup> Shaffer (8) pp. 147-150, including Table II. Only the lower two-thirds of this table applies to our problem. The non-protein respiratory quotients referred to in our text were computed by the ordinary method, and not by the special calculation described by Shaffer.

num correction, which would be in full force only if the control stored none of the glycerol and the patient all of it.

TABLE 1

*Diets.*

Date.	Protein.	Fat.	Carbohydrate.	Calories.	N <sub>2</sub> in urine.	Remarks.
(1)	(2)	(3)	(4)	(5)	(6)	(7)

## Franklin K.

Feb., 1916	gm.	gm.	gm.		gm.	
1	34	130	242	2343	8.0	
2	96	118	265	2577	11.8	
3	92	100	271	2411	13.3	
4	88	93	239	2203	13.3	
5	93	107	233	2325	13.8	
6	96	132	294	2821	15.5	
7	99	147	305	3019	15.8	
8	95	146	334	3119	13.6	
9	94	166	265	3014	14.5	
10	93	170	355	3423	13.3	Last meal at 5.30 p.m.
11	94	128	273	2692	14.2	After calorimeter observation. Last meal at 8.45 p.m.
12	15	2	8	106	11.4	
13	0	0	0	0	13.1	Broth and thrice boiled vegetables.
14	37	69	203	1626	13.5	Taken after calorimeter observation.

## Henry D.

Jan., 1924						
4	100	200	400	3910	9.7	
5	100	217	400	4068	10.3	
6	100	200	400	3910	11.3	
7	100	200	400	3910	8.0	
8	100	200	500	4320	14.3	
9	100	200	505	4340	14.7	
10	100	303	263	4308	11.0	Last meal at 11.40 a.m. Calorimeter observation 14 hrs. later.

TABLE I—*Concluded.*

Date.	Protein.	Fat.	Carbohydrate.	Calories.	N <sub>2</sub> in urine.	Remarks.
(1)	(2)	(3)	(4)	(5)	(6)	(7)

## Henry D.—Continued.

<i>Jan., 1924</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>		<i>gm.</i>	
17-20	100	200	300	3502	15.3†	
21	100	198	400	3892	17.8	
22	100	200	400	3909	15.3	
23	69	138	248	2576	13.3	
24	99	197	400	3880	16.1	
25	92	192	354	3616	15.3	
26	100	200	400	3910	13.0	
27	67	134	266	3005	11.3	Last meal at 11.30 a.m.
28*	0	0	0	0	9.0	Calorimeter observation.
29	0	0	0	0		“ “

\* Two eggs and bacon taken at 4 p.m. but promptly vomited.

† Determined on Feb. 20.

*Apparatus and Subjects.*

Since no apparatus was available for the continuous measurement of the respiratory exchange over a period of days, it was necessary to rely on repeated observations 2 to 3 hours in length. From the data thus obtained the quantity of carbohydrate oxidized per hour was calculated according to the method described by Lusk ((9) chapter 1), using, however, his more recent modification of the tables of Zuntz and Schumburg (10). A critique of the respiratory quotient as an index of the metabolism of carbohydrate may be found elsewhere (11). The quantity of carbohydrate oxidized per hour was plotted against the time in hours elapsed since the last meal. The area under the curve between the first and last observations was taken as an index of the glycogen in storage at the start (see Fig. 1). This curve did not include the first 15 to 18 hours, this interval being allowed for the removal of carbohydrate other than glycogen, which might have been present as a result of the last meal.

In order to make sure that the glycogen deposits were filled to capacity, both patients were given high caloric diets rich in carbohydrate, as may be seen in Table I. After the first observation Henry D. resumed the diet of January 10 for a week, and then took the food shown in the table.

The exact intake of the subject L. prior to the fast was not recorded, but it is evident from his dietary ((6) p. 32) that he took an abundance of carbohydrate and total calories.

The respiration calorimeter of the Russell Sage Institute of Pathology was used. The apparatus was tested by means of alcohol checks, which may be found in Papers XXV (12) and XXXIX (11) of this series. Patients were kept under close observation in the metabolism ward.

### *Case Histories.*

Owing to the severe ketosis produced in exophthalmic goiter by fasting we hesitated to subject any more patients to this procedure, although the study of a greater number, especially those with the more severe type of the disease, would have been desirable. Both of the following patients presented typical cases of a moderate degree of severity.

Franklin K., male, age 39 years, had a height of 170.1 cm. and a weight of 41.1 kilos. His symptoms were weakness, nervousness, palpitation of the heart, shortness of breath, indigestion, and loss of weight. Examination revealed pallor, exophthalmos, rapid pulse, and marked sweating. His basal heat production was 55.2 calories per sq. meter per hour or plus 40 per cent according to the standards of Aub and Du Bois (13).

Henry D., male, age 24 years, had a height of 171.6 cm. and a weight of 57.4 kilos. His symptoms were palpitation, excessive appetite, nervousness, diarrhea, and difficulty in swallowing. Examination revealed exophthalmos, enlargement of thyroid, rapid pulse, and tremor of hands. His basal heat production in the calorimeter was 56.0 calories per sq. meter per hour or plus 42 per cent according to the standards mentioned. Other basal determinations were made with the Roth-Benedict apparatus, the highest being plus 68 per cent.

### *Results.*

The more important of the calorimeter data are shown in Table II, and those taken from Benedict (6) in Table III. From these the quantities of carbohydrate oxidized per hour have been cal-

culated and appear in Column 6 of Table III. They have also been plotted in Fig. 1 against the time in hours. As may be readily seen, Curves 1 and 2 representing the patients are higher than in the case of the control, indicating that they had a larger supply of glycogen at the start. The area under Curve 1 from 19 to 64 hours is an index of the quantity of glycogen originally present in Franklin K., and totals 162 gm. as against 117 gm. for the control, measured over the same period. Similarly, Henry D. oxidized from the 14th to the 51st hour 183 gm. of carbohydrate, as against 121 for the normal, again indicating a much greater store of glycogen at the start. The figures are detailed in Table IV.

In Fig. 1, Curve 2, 26th hour, there is a peak instead of the steady decline which might be expected with the progress of the fast. A possible explanation may be found in the fact that the patient, Henry D., was very restless, with the result that his metabolism was increased to 103 per cent above normal. This tended to stimulate the oxidation of carbohydrate along with other substances, and to remove glycogen which might otherwise have remained in storage. Perhaps it would be more accurate to calculate the quantity of carbohydrate which would have been oxidized, had the total calories remained at the initial level of 94 (Table II, Column 7). The percentage of calories from carbohydrate was 20 which multiplied by 94, gives 18.8 calories, or 4.6 gm. Similarly, on the last day, the percentage was 9.7, hence the corrected carbohydrate was 2.2 gm. If these values are plotted on the graph instead of the second and third points of Curve 2, it will be seen that they are likewise above the level of the control. Even with this correction no diminution in glycogen deposits can be inferred.

For a control we have used only the subject L. although other data are available. The normal fasting men studied at an earlier date by F. G. Benedict (14) oxidized on the average 110 gm. of carbohydrate the 1st day, 40.3 the 2nd, and 21.8 the 3rd, a total of 172 gm. Apparently all the observations began 13 hours after the last meal. Extrapolating the curve of the subject L. to include the same period, gives 160 gm. oxidized, or 12 gm. less than the normal average. This individual, may therefore be considered as normal in regard to his ability to store glycogen.



TABLE II.  
Calorimeter Data in Terms of Averages per Hour.

Data, weight, surface area.	Time of obser- vation.	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	N <sub>2</sub> in urine.	Indirect calorim- etry.	Total metab- olism above nor- mal.*	Average pulse.	Work added.†	Remarks.
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Franklin K.										
Feb. 11, 1916 41.1 kg. 1.41 sq. m.†	11.35 a.m.- 1.35 p.m.	25.9	23.4	0.804	0.402	77.9	+39	118	19	Fairly quiet; no food for 19 hrs.
Feb. 14, 1916 39.2 kg. 1.39 sq. m.†	11.50 a.m.- 1.50 p.m.	24.5	23.7	0.750	0.617	77.5	+40	123	19	Quiet; no food for 64 hrs.
Henry D.										
Jan. 11, 1924 57.4 kg. 1.68 sq. m.	12.13 a.m.- 3.13 a.m.	30.3	28.4	0.777	0.389	94.0	+42	100	28	Restless during 3rd hr., otherwise quiet; no food for 14 hrs.
Jan. 28, 1924. 54.0 kg. 1.63 sq. m.	12.31 p.m.- 2.31 p.m.	41.7	39.5	0.769	0.304	131.0	+103	122	90	Very restless; no food for 26 hrs.

Jan. 29, 1924.	12.54 p.m.-	37.5	36.3	0.751	0.747	119.1	+86	137	69	Restless; no food for 51 hrs. §
53.1 kg.	3.54 p.m.									
1.62 sq. m.										

\* *I.e.*, above normal standard according to Aub and Du Bois (13).

† The work adder is an instrument by which the activity of the patient is recorded. A very quiet patient raises the work adder less than 5 cm. and a very restless one more than 25 cm.

‡ Linear formula.

§ Had two eggs and bacon at 4 p.m. Jan. 28, but vomited it.

**TABLE III.**  
*Carbohydrate Oxidized per Hour While Fasting.*

Time of observation.	Length of fast.*	CO <sub>2</sub> per min.	O <sub>2</sub> per min.	Urinary nitrogen in 24 hrs.	Carbohydrate oxidized per hr.
(1)	(2)	(3)	(4)	(5)	(6)
Normal subject L.					
<i>1918</i>	<i>hrs.</i>	<i>cc.</i>	<i>cc.</i>	<i>gm.</i>	<i>gm.</i>
Apr. 14 8.30 a.m.-9.30 a.m.	15	182	223	7.10†	5.20‡
Apr. 14-15 10 p.m.§-8 a.m.	33	165¶	212¶	7.10†	2.90‡
Apr. 15-16 10 p.m.§-8 a.m.	57	159¶	211¶	8.40†	1.63‡
Apr. 16-17 10 p.m.§-8 a.m.	81	151¶	206¶	11.34†	0.16‡
Apr. 17-18 10 p.m.§-8 a.m.	105	150¶	202¶	11.87†	0.62‡
Franklin K. Exophthalmic goiter.					
<i>1918</i>					
Feb. 11 11.35 a.m.-1.35 p.m.	19			9.6	5.71
Feb. 14 11.50 a.m.-1.50 p.m.	64			14.8	1.52
Henry D. Exophthalmic goiter.					
<i>1924</i>					
Jan. 11 12.13 a.m.-3.13 a.m.	14			9.6	4.86
Jan. 28 12.31 p.m.-2.31 p.m.	26			7.3	6.42
Jan. 29 12.54 p.m.-3.54 p.m.	51			17.9	2.81

\* From the time of the last meal to the middle of the respiratory observation.

† From Table 27 of Benedict's monograph (6).

‡ Calculated from the data of Columns 3, 4, and 5.

§ Approximate time at which observation was begun.

|| From Table 46 of Benedict's monograph (6).

That the normal is subject to wide variations has been shown by Schoendorff (15) for the dog, and by Benedict (14) for man.<sup>2</sup> What the glycogen content of our patients would have been in the absence of exophthalmic goiter is not known. This point could be settled by a study in which the glycogen content is measured in one and the same patient before and after operative treatment.

Although the oxidation of carbohydrate was not diminished in exophthalmic goiter, it was apparently insufficient to prevent the onset of ketosis. After 51 hours of fast Henry D. excreted acetone bodies at the rate of 19.4 gm. per day. According to the theory of the ketogenic balance this should be associated with a large percentage of fat in the metabolism, as was indeed the case, judging from his respiratory quotient of 0.75. Estimations of ketones are not available in the case of Franklin K. but the ammonia

TABLE IV

Subject.	Estimated glycogen deposit.
	<i>gm.</i>
Franklin K. Exophthalmic goiter . . . . .	162
L., normal . . . . .	117
Henry D. Exophthalmic goiter . . . . .	183
L., normal . . . . .	121

nitrogen of the urine was 0.566 gm. higher on the day of the last observation than it was 2 days earlier (0.925 as compared to 0.359). If this increase were due to ketosis, it would correspond to 2.3 gm. of keto acid, expressed as acetone, or several times the normal output. This again was consistent with the respiratory quotient, which happened to be the same as in the former patient, *i. e.* 0.75.

In both of the patients as well as in the control, the elimination of nitrogen was less on the 1st day of the fast than on the succeeding days (Table III). Lusk ((9) pp. 72, 89) has pointed out that

<sup>2</sup> In children, however, S. Z. Levine has found the glycogen stores under suitable conditions to be remarkably constant as between different individuals. In four subjects he found the reserve per sq. meter of body surface to be within  $\pm 3$  per cent of the average (personal communication). It is possible that the wide variations noted by Benedict are due to differences in diet or exercise.

the greater the glycogen reserves, the less the excretion of nitrogen, the explanation being the well known protein-sparing action of carbohydrate. In our patients, therefore, the relatively low nitrogen excretion of the 1st day is confirmatory evidence of the presence of glycogen in storage.

#### DISCUSSION.

According to the results detailed above there is no necessary reduction of the glycogen deposits in exophthalmic goiter. Either there is no fault in the mechanism by which glycogen is stored, or else the defect is too trifling to resist the mass action of a diet rich in carbohydrate. Still another possibility presents itself; namely, that the organism is, so to speak, a spendthrift of glycogen—easy come, easy go, as the saying is. In other words, the body may be able to store carbohydrate with ease, but in such unstable equilibrium that it is readily discharged into the blood stream for consumption by the tissues. This theory is not excluded by the evidence; indeed, it appears to fit the two salient facts, the ample glycogen deposits, and the rapid oxidation of carbohydrate.

Our conclusions seem at first glance to be at variance with the well established fact that thyroid gland when given to animals reduces the glycogen of the liver. This may, however, be due not to a defect in storage, but to increased oxidation. Both of these possibilities have been considered by the authors cited above. Parhon (5) ascribed the depletion of glycogen deposits in part, at least, to increased metabolism, Kuriyama (4) did not commit himself definitely, one way or the other, while Cramer and Krause (3) took the view that the storage of glycogen was defective, basing their conclusion on the idea that the active substance of the thyroid gland tends to depress the oxidation of carbohydrate. Evidence has since been obtained by Du Bois (2) and by Sanger and Hun (1), that this is not the case in exophthalmic goiter. These results were confirmed for rats by Cramer and M'Call (16, 17, 18, 19), who found an actual increase in the oxidation of carbohydrate as the result of feeding with thyroid gland. They argue that this is the effect, rather than the cause of the disappearance of glycogen from the liver. In other words, they postulate a primary action of the extract on the glycogenic function of the liver, and a secondary increase in the metabolism of

carbohydrate. They give, however, no direct evidence of this primary defect, and our observations go to show that it did not exist with our two patients. Should the results be confirmed for exophthalmic goiter in general the theory of Cramer and M'Call would not be tenable for this disease.

Defective storage of glycogen is not the only possible explanation for the behavior of the blood sugar curve following the ingestion of glucose. The increase in the height and breadth of the curve noted by Sanger and Hun (1) might be due to an unusually rapid or complete absorption of sugar from the gut, or to a temporary increase in glycogenolysis.

In support of their theory these authors cite the clinical observation that patients with exophthalmic goiter often develop ketosis after brief periods of fasting. This fact was well shown by our patient, Henry D., who after 51 hours excreted acetone bodies at the rate of 19.4 gm. a day. This occurred, however, in spite of the fact that the oxidation of carbohydrate was as great as in the normal subject. Judging from the respiratory quotient of 0.75, more fat was oxidized along with the carbohydrate than could be taken care of by the latter. This, rather than a small glycogen reserve, appears to be the cause of ketosis.

Regardless of the cause, the ketosis was very striking in degree, and demonstrates the fact that carbohydrate starvation is not a procedure lightly to be undertaken in this disease.

It has been stated above that ketosis occurred with a respiratory quotient of 0.75. This happens to be the average level at which it occurs in other conditions (20, 21). If this should prove to be a constant finding in exophthalmic goiter it would indicate that there is no disturbance in the mechanism of the ketogenic balance in this disease.

#### SUMMARY AND CONCLUSIONS.

1. The glycogen reserves of two patients with exophthalmic goiter were estimated to be at least as great as normal.
2. This is evidence against the theory that there is any defect in the mechanism by which glycogen is stored.

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## ANTIRICKETIC SUBSTANCES.

### III. THE CATALYTIC FORMATION OF AN ANTIRICKETIC CHOLESTEROL DERIVATIVE.

By CHARLES E. BILLS.

With the cooperation of FRANCIS G. McDONALD.

(From the Research Laboratory, Mead Johnson and Company, Evansville, Indiana.)

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It has been demonstrated by Hess, Weinstock, and Helman (1), Rosenheim and Webster (2), and Steenbock and Black (3) that under the influence of ultra-violet rays cholesterol is transformed into an antiricketic modification. In a previous communication (4) I have shown by a reaction with *n*-butyl nitrite that in one respect the activated cholesterol behaves chemically the same as the antiricketic vitamin.

Now the researches of Windaus (5), Dorée and Gardner (6), Schrötter and Weitzenböck (7), and Mauthner (8) would indicate that cholesterol is an olefinic terpene. If such is the case it is not strange that the molecular structure of cholesterol should be altered by physical agents, light among others. To illustrate: The olefinic structure in cholesterol appears to be a vinyl radical, and Klatte and Rollett (9) observed that vinyl compounds polymerize under ultra-violet rays. Moreover, the terpene structure appears to be related to pinene, which according to Urbain and Scal (10) is extensively altered by a metallic arc.

The light-sensitive groups in cholesterol—the olefine and terpene groups—are subject also to polymerization by the catalytic action of floridin.<sup>1</sup> Gurvich<sup>2</sup> (11), Venable (12), and Lebedev and Filonenko (13) studied the action of floridin on pinene and other ter-

<sup>1</sup> A fuller's earth from northern Florida possessing marked catalytic properties.

<sup>2</sup> I am indebted to Dr. L. J. Pessin of the Johns Hopkins University for translating the work of Gurvich from the Russian.

penes, and on amylene and other olefines. In brief, they found the effect to consist of polymerization, followed by degradation of the polymers. Of particular significance is the observation of Kawai and Kobayashi (14) that cholesterol when fused with "Japanese acid clay" yields fluorescent, petroleum-like hydrocarbons.

Following the analogy suggested by the above work, I investigated the action of floridin on cholesterol. The catalyst was obtained as a yellowish white, "200 mesh" powder from the Jamieson, Florida, mine of the Floridin Company. It contained 19.1 per cent of water expellable at 880°.

Some floridin was activated by heating until the moisture content was reduced to 8.9 per cent. When cholesterol was fused with this product the ensuing reaction was uncontrollable, and I therefore proceeded to experiment with cholesterol dissolved in various solvents. 2.50 gm. of the activated floridin were added to 0.50 gm. of cholesterol dissolved in 15 ml. of xylene. The mixture was refluxed for 2 hours, a Kjeldahl flask being used to minimize the effects of violent bumping.

Almost immediately the suspension darkened in color. In 5 minutes it was deep purple; in 15 minutes, blue; and after 30 minutes it remained a dark greenish blue. The suspension after 2 hours was filtered. The filtrate was yellow with a blue fluorescence. The floridin residue was washed repeatedly with xylene and with benzene, but no pigment passed into solution except a little of the yellow substance which appeared to have been firmly adsorbed on the floridin. However, it was found that ethyl ether or acetone would release the pigment and restore the floridin to its normal color. The completely extracted pigment was not greenish blue, but a deep orange color! Evidently the greenish blue appearance was a physical effect, associated with the adsorption of the pigment in the pores of the floridin.

The combined xylene and benzene filtrates and ether extracts were evaporated to dryness by gentle warming. There remained an odorous, orange resin. That the resin certainly is a product of the action of floridin on cholesterol was demonstrated by refluxing xylene with floridin but without cholesterol, and with cholesterol without floridin; there was no reaction in either case.

Inasmuch as the boiling point of xylene is about 139°, I decided

to investigate the action in solvents which can be refluxed at lower temperatures. 2.50 gm. of activated floridin, 0.50 gm. of cholesterol, and 15 ml. of toluene (b.p. 110°) were refluxed 2 hours as before. The color changes in the floridin suspension occurred a little more slowly than with xylene, and at the end of 2 hours the color was a deep blue. The toluene filtrate was yellow with a blue fluorescence. The ether extract upon evaporation left an odorous, orange resin.

A similar preparation was then made with benzene (b.p. 80°). The color changes in the floridin suspension were decidedly slower than with toluene, so that after 2 hours only the purple stage had been reached. The benzene filtrate was pale yellow and slightly fluorescent. The extracted resin possessed little odor, and was lighter in color than the product from toluene.

A similar preparation was made with carbon tetrachloride (b.p. 76°). The color changes in the floridin suspension were even slower than with benzene, although in 2 hours the purple stage was reached. However, the carbon tetrachloride filtrate and the ether extracts were somewhat more colored than those with benzene. The fluorescence was nil, and the resin after evaporation was odorless.

From the foregoing experiments one might assume that the rate of catalytic alteration of cholesterol dissolved in various solvents depends upon the temperature at which the solvent is refluxed. However, this is not true in all cases. There is a remarkable specificity in the behavior of solvents, so that in certain solvents there is no catalysis, even though the boiling point be not particularly low. 0.50 gm. of cholesterol were dissolved in 15 ml. of each of the following solvents: ethyl acetate (b.p. 77°), absolute ethyl alcohol (b.p. 78°), *n*-propyl alcohol (b.p. 97°), and isobutyl alcohol (b.p. 107°). To each solution 2.50 gm. of activated floridin were added, and the suspensions refluxed for 2 hours as before. In no case was there any color change, and from the filtrates crystals having the melting point of unaltered cholesterol were obtained.

An investigation of the chemical changes given by floridin and other catalysts with cholesterol will be reported later, but for the present it will suffice to describe some biological tests with one of the reaction mixtures. To this end a cholesterol-carbon tetrachloride-floridin preparation was made under carefully regulated

conditions. A second portion of floridin was activated by heating for 2 hours at  $280^{\circ} \pm 5^{\circ}$ . The moisture content was reduced to 4.7 per cent, as compared with 8.9 per cent in the first activation. Incidentally, the second sample was much the more active, for it gave the series of color changes almost three times as rapidly. The cholesterol was a specially purified product, the preparation of which I have described elsewhere (4).

1.25 gm. of cholesterol dissolved in 37.5 ml. of carbon tetrachloride were refluxed for 5 hours with 6.25 gm. of activated floridin. The color changes in the floridin suspension (indicative of the rate and extent of reaction) were: 5 minutes, noticeable darkening; 15 minutes, old rose; 45 minutes, reddish purple; 2 hours, bluish purple; 3 hours, purplish black; 5 hours, black. The suspension was filtered, and the floridin residue extracted with ether and acetone until no more coloring passed into solution. The combined filtrate and extracts exhibited a deep orange color with a slight fluorescence.

In testing antiricketic concentrates or irradiated cholesterol, I have lately been adding to these preparations small amounts of Newfoundland seal oil which serves the purpose of dissolving and protecting the antiricketic material and assisting its trituration with the diet. 5 gm. of seal oil were added to the combined filtrate and extracts, and the mixture was gently evaporated onto a sufficient quantity of McCollum's Diet 3143 to make 250 gm. of a modified ration containing 0.5 per cent of catalyzed cholesterol and 2 per cent of seal oil. The diet was administered to five rickety rats for 5 days. The Shipley line test was performed on the tibias and it showed advanced healing of the ricketic lesions (Table I).

Two control preparations were made. In the first, 1.25 gm. of cholesterol were refluxed 5 hours with 37.5 ml. of carbon tetrachloride without any floridin. There was no color change. 5 gm. of seal oil were added and the mixture evaporated onto Diet 3143 as before. No healing was induced, which shows that the antiricketic substance is not produced in the absence of floridin. In the second control 6.25 gm. of activated floridin were refluxed for 5 hours with 37.5 ml. of carbon tetrachloride without any cholesterol. There was no color change. The floridin was filtered and extracted, the seal oil was added, and the modified diet ad-

ministered as before. No healing was induced, which shows that the antiricketic substance is not produced in the absence of cholesterol.

TABLE I.

For convenience in tabulation, the quality of healing observed by the line test is here expressed on a scale of 4 degrees. One plus sign (+) indicates a just perceptible healing; two plus signs (++), a distinct healing; three plus signs (+++), an advanced healing; and four plus signs (++++), a practically complete healing. The reader should realize that these values bear no numerical relation to each other.

Rat No.	Sex.	Preparation administered.	Grade of test.	Weight.	Average daily consumption.
				gm.	gm.
1677	M.	Diet 3143 control.	—	94-98	8.4
1678	F.	" 3143 "	—	72-75	6.8
1679	"	" 3143 "	—	70-72	6.8
1680	M.	" 3143 "	— (?)	92-93	7.4
1681	F.	" 3143 "	—	69-71	7.6
1682	M.	Catalyzed cholesterol $\frac{1}{2}$ per cent plus seal oil 2 per cent.	+++	81-87	8.2
1683	"	" " "	++	73-74	6.4
1684	F.	" " "	+++	77-83	7.2
1685	M.	" " "	+++	72-74	6.6
1686	"	" " "	+++	78-81	8.0
1628	F.	Cholesterol $\frac{1}{2}$ per cent refluxed without floridin, plus seal oil 2 per cent.	—	68-75	7.8
1612	M.	" " "	—	83-83	7.0
1611	F.	" " "	—	70-72	5.8
1610	"	" " "	—	78-82	7.6
1609	"	" " "	—	75-77	6.6
1613	F.	Floridin refluxed without cholesterol, extracted, plus seal oil 2 per cent.	—	70-72	7.2
1614	"	" " "	—	84-84	8.6
1615	"	" " "	—	69-69	7.0
1617	M.	" " "	—	72-72	7.0
1651	F.	" " "	—	77-77	8.6

The foregoing experiments should not be misinterpreted. They do not prove that the antiricketic substance present among the

products of the catalytic alteration of cholesterol is identical with the antiricketic vitamin, or even with the antiricketic cholesterol modification produced by irradiation. Either of these possibilities may be true, but it is equally possible that a new antiricketic derivative was formed, or that the healing resulted from a systemic disturbance following mild poisoning by one of the evidently numerous reaction products.

#### SUMMARY.

1. When a solution of cholesterol in xylene, toluene, benzene, or carbon tetrachloride is refluxed with activated floridin a catalytic action takes place, resulting in the formation of fluorescent and resinous products.

2. There is a specificity among solvents in accordance with which no catalysis occurs in ethyl acetate, ethyl alcohol, *n*-propyl alcohol, or isobutyl alcohol.

3. An antiricketic substance of unknown nature was demonstrated among the products of the action of floridin on cholesterol in carbon tetrachloride.

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## ACETYL MONOSES. I.

By P. A. LEVENE AND HARRY SOBOTKA.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, January 23, 1926.)

The first paper of this series is the outcome of continued investigations into the causes of the abnormalities in the optical behavior of mannose and its derivatives. The work necessitated an analysis of the existing data on the acetylated monoses generally and incidentally on the partially methylated sugars also. The survey of the existing data led to some comprehensive deductions applicable to sugars generally and useful for the interpretation of the peculiarities in the optical conduct of mannose and of its derivatives. For the convenience of the reader it was decided to present the theoretical deduction in the form of a separate introductory paper and to present the experimental material in separate papers as the work progresses.

The peculiarities in the physical properties of each monose are determined by its configuration, by the character of the ring structure, and by the allocation of the groups on carbon atom (1) with respect to the groups on carbon atom (5). The knowledge of the configuration of all known monoses has been furnished by the classical work of Fischer and his coworkers. Information as to the configuration on carbon atom (1) has been advanced principally by the work of Hudson. The question of the ring isomerism of individual monoses is at present one of the most important problems of sugar chemistry.

If a sufficient number of the "lactal"<sup>1</sup> isomers of individual sugars were known, it would be possible to disclose optical properties peculiar to each ring structure, and with this information

<sup>1</sup> The term "lactal" for the ring of the sugars was suggested by Helferich, B., and Fries, F. A., *Ber. chem. Ges.*, 1925, lviii, 1246.



the ring structure of any new sugar could be established. In recent years Bergmann,<sup>2</sup> Haworth,<sup>3</sup> Pryde,<sup>4</sup> and also Levene and Meyer<sup>5</sup> have brought out the fact that the ordinary glucosides of fructose, galactose, and mannose possess a lactal structure different from  $<1, 4>$ . The question now arises whether the ring structure in the sugars is the same as in the glucosides from which they are derived. Two experimental facts discovered in recent years should be recalled in this connection. Fischer<sup>6</sup> and later others have established the fact that when a partially substituted polyhydric alcohol is further substituted the original groups may wander to new positions. Second, the ring formation in aldoses is analogous to the lactone formation in sugar acids. For the lactones Levene and Simms<sup>7</sup> have demonstrated that the free acid in solution is in equilibrium with more than one lactone. These lactones differ in the size of their rings and the possibility of separation in crystalline state of one or the other lactone is determined not only by the so called "stability" of the lactone but mainly by its solubility. Thus, accepting that a sugar in solution is capable of existing in at least four forms (two mutamers of a five-membered lactal ring and two of the six-membered) it is of course impossible to predict which form will crystallize. It is unfortunate that only a few simple sugars have been obtained crystalline in two forms and none in more than two.

Among the acetyl derivatives of sugars, the number of known isomers is greater. This fact offers an opportunity to observe relationships between physical properties and ring structure. From these relationships some general rules can be formulated which later may serve to explain the ring structure of simple sugars in general.

The acetyl derivatives which will be discussed in this place are:

- (a) completely acetylated monoses; (b) (1)-methoxy acetates; (c) (1)-halogen acetates.

<sup>2</sup> Bergmann, M., *J. Chem. Soc.*, 1923, cxxiii, 1277.

<sup>3</sup> Haworth, W. N., and Linnell, W. H., *J. Chem. Soc.*, 1923, cxxiii, 294.

<sup>4</sup> Pryde, J., *J. Chem. Soc.*, 1925, cxxvii, 1808.

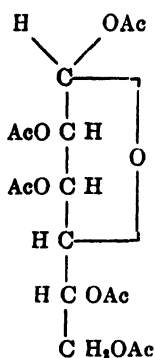
<sup>5</sup> Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1924, lx, 167.

<sup>6</sup> Fischer, E., *Ber. chem. Ges.*, 1920, liii, 1621.

<sup>7</sup> Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1925, lxxv, 31.

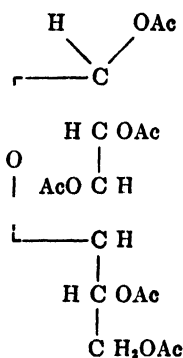
*Pentacetates of Hexoses and Hexacetates of Heptoses.*

In this group of substances representative members of at least four types are known. Two of them have five-membered and two are supposed to have six-membered ring structures. Each pair is distinguished by the direction of the ring with respect to the carbon chain. In projection the ring will be represented either to the right or to the left of the chain. Examples of ring structures differing in this respect are the butyleneoxidic rings of mannose, galactose, and  $\alpha$ -glucoheptose of which the  $\alpha$ -forms are represented as follows:



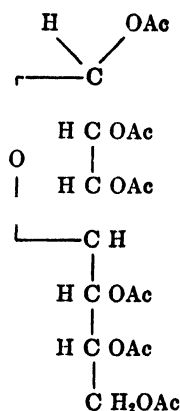
I.

*d*-Mannose  
pentacetate.



II.

*d*-Galactose  
pentacetate.

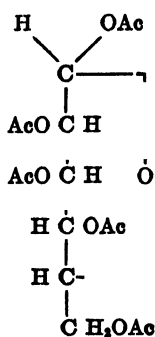


III.

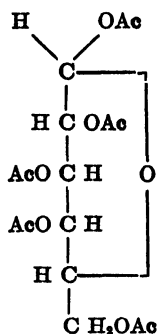
*d*- $\alpha$ -Glucoheptose  
hexacetate.

Also, the amylenoxidic forms are capable of existing in two types. In *d*-hexoses, however, the amylenoxidic rings must all face to the right, inasmuch as according to Rosanoff's<sup>8</sup> definition of *d*-sugars the hydroxyl group attached to carbon atom (5) is always in the same position. In *d*-heptoses this ring may be either to the right or to the left. Thus glucoheptose would have an amylenoxidic (six-membered) ring to the right whereas galactoheptose would have a six-membered ring to the left.

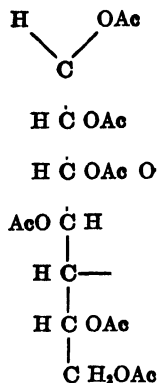
<sup>8</sup> Rosanoff, A., *J. Am. Chem. Soc.*, 1916, xxviii, 814.



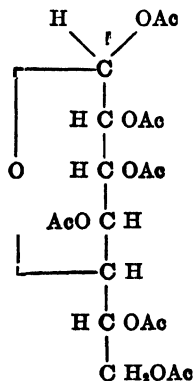
IV.

*d*-Mannose  
pentacetate.

V.

Galactose  
pentacetate.

VI.

*d*- $\alpha$ -Glucoheptose  
hexacetate.

VII.

*d*- $\alpha$ -Galactoheptose  
hexacetate.

It is now important to inquire whether data can be found which would permit a choice between the five- and six-membered ring structures as well as between the right and left side ring structures independent of the size of the ring.

*Differentiation of the Five- and Six-Membered Ring Structures.*

If the numerical values of the molecular rotations of carbon atom (1) are calculated according to the rule of Hudson the results shown in Table I are obtained.

From this table it is apparent that carbon atom (1) of the pentacetates of glucose, glucoheptose, and of the  $\gamma$ - and  $\delta$ -forms of galactose have the same numerical value and that of the  $\alpha$ - and  $\beta$ -forms of the pentacetates of galactose and of mannose also have identical values. Since the two pairs of pentacetates of galactose differ in their ring structures, the numerical value of the rotation of carbon atom (1) may be considered as a characteristic function of the ring. To the pentacetate of mannose, then, the same ring structure may be attributed as to the common form of galactose.

TABLE I.  
*Molecular Rotations of Sugar Pentacetates.*

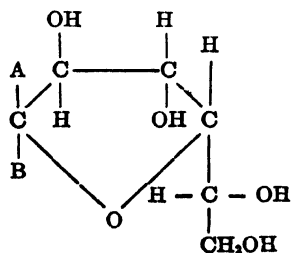
	$[M]_D \alpha \cdot 100$	$[M]_D \beta \cdot 100$	Difference. (2 A).
<i>d</i> -Glucose pentacetate.....	+39,600	+1,500	+38,100
<i>d</i> - $\alpha$ -Glucoheptose hexacetate.....	+40,200	+2,200	+38,000
Third and fourth <i>d</i> -galactose pentacetate.	-16,400*	+23,800†	+40,200
First and second <i>d</i> -galactose pentacetate.	+41,700	+9,000	+32,700
<i>d</i> -Mannose pentacetate.....	+22,500	-9,700	+32,200

\*  $\gamma$ -form.

†  $\delta$ -form.

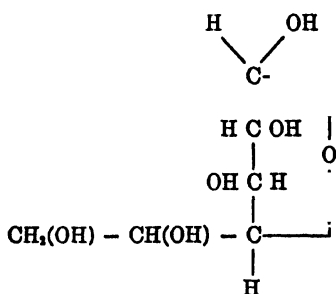
Since the common forms of methyl galactoside and the common forms of methyl mannoside possess a six-membered ring structure, it may be permissible to attribute the six-membered ring structure also to the common forms of galactose pentacetates and to the mannose pentacetates.

A spatial representation similar to that commonly used for hydroaromatic compounds would give the following figure (VIII):

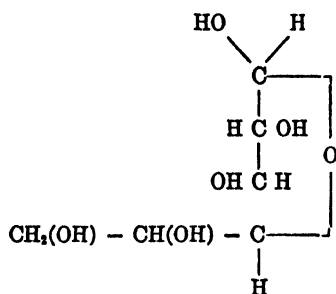


(A = OH in  $\alpha$ -glucose  
B = OH in  $\beta$ -glucose.)

VIII.



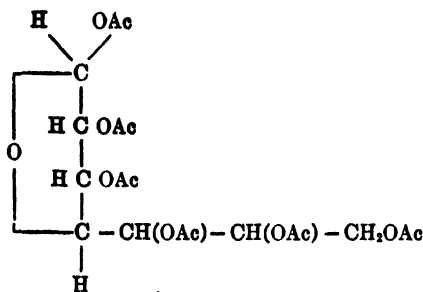
VIII a.  
 $\alpha$ -Glucose.



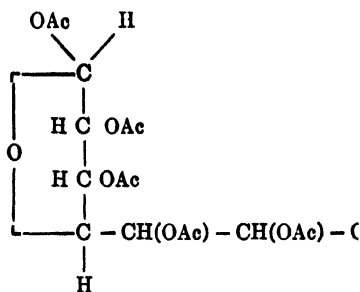
VIII b.  
 $\beta$ -Glucose.

In figure VIII, A and carbon atoms (5) and (6) are located on opposite sides of the plane of the ring. On the other hand, B is located on the same side of the plane of the ring. In projection the two forms are presented in figures VIIIa and VIIIb. The two forms resemble the *cis* and *trans* configurations of fumaric and maleic acids.

*Hexacetates of Glucoheptose.*—The projections of the  $\alpha$ - and  $\beta$ -forms of this sugar are represented by the following figures:



X a.  
 $\alpha$ -Form.  
m.p. 164°C.



X b.  
 $\beta$ -Form.  
m.p. 135°C.

In this instance the  $\alpha$ -form has the *cis* configuration and the  $\beta$ -form the *trans* configuration. In this case the  $\alpha$ -form was found to have the higher melting point.

Furthermore, it is known that the  $\beta$ -pentacetates of galactose, xylose, mannose, etc., are readily transformed into the  $\alpha$ -forms

and that the reverse process is much slower so that in equilibrium the  $\alpha$ -form predominates. In the case of the glucoheptose hex-acetates, however, the specific rotation of a solution of  $\beta$ -hexacetate was found by Hudson and Yanowsky<sup>9</sup> to increase in the presence of zinc chloride only to  $+25^\circ$  and several recrystallizations were

TABLE II.  
*Molecular Rotations and Melting Points of Some Alkyl Monosides.*

	$\alpha$ -Form.		$\beta$ -Form.		Difference $\alpha - \beta$ .
	m.p.	100 $[M]_D$	m.p.	100 $[M]_D$	
Diacetone methyl mannosides.....		+9,600*	37   -11,600*		21,200
Tetracetylmethyl glucosides.....	100°	+47,300	104-105   -6,600		53,900
Tetracetylmethyl galactosides.....		+25,100	94   -3,800		28,900
Tetracetylmethyl mannosides.....	65°	+17,700		-1,100†	18,800†
Tetracetylmethyl $\gamma$ -mannoside‡.....			105   -11,000		27,000§
Tetracetylethyl mannosides.....		+16,000	111-113   +700		15,300
Tetracetylethyl $\gamma$ -mannoside.....			81-82   -10,400		26,400§

\* In acetylenetetrachloride. (The  $[\alpha]_D$  for the  $\alpha$ -form is given by Pringsheim (Pringsheim, H., *Zucherchemie*, Leipzig, 1925) erroneously as  $+23$ , whereas the pure substance has  $+34.9$ . Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1924, lix, 145.)

† Approximated extrapolation from  $\beta$ -ethyl derivative.

‡ The form prepared by Dale by the action of alcoholic HCl on the  $\gamma$ -form is not discussed in this paper.

§  $[M]_{D\alpha} - [M]_{D\gamma}$ .

necessary to obtain the pure  $\alpha$ -form of  $[\alpha]_D = +87^\circ$ . Thus it seems that as a general rule after the action of zinc chloride, not necessarily the  $\alpha$ -form predominates in equilibrium, but the trans form, which in the case of the glucoheptoses on account of the left side ring is the  $\beta$ -form.

<sup>9</sup> Hudson, C. S., and Yanowsky, E., *J. Am. Chem. Soc.*, 1916, xxxviii, 1575.

obtained regardless of the  $\alpha$ - or  $\beta$ -structure of the parent pentacetate. In the past, the  $\beta$ -structure was attributed to the (1)-halogen acetyl sugars, for the reason that, as a rule, they led to  $\beta$ -glucosides. Recently, Hudson<sup>13</sup> calculated the numerical values of the rotations of carbon atoms (1) in a series of (1)-halogen acetyl derivatives of sugars and on the basis of their dextrorotation assigned the  $\alpha$ -structure to this class of derivatives.

It seems to the writers that there still exist valid grounds in favor of the older view. They are the following:

1. It was shown by Levene and Mikeska<sup>14</sup> that configurationally related alcohols and halides may rotate in opposite directions. Clough<sup>15</sup> reached the conclusion that configurationally related  $\alpha$ -hydroxy and  $\alpha$ -halogen acids rotate in opposite directions. In a comprehensive way the rule may be formulated that the substitution of a hydroxyl by a halogen or an  $-SH$  group by  $-SO_2OH$  leads in the *d*-series to a drop in dextrorotation and in the *l*-series to a drop in levorotation.

2. Wrede<sup>16</sup> has shown that oxidation of his thiodisaccharide of the trehalose type ( $\alpha, \alpha'$ ) to the corresponding sulfone led to a drop in dextrorotation.

3. Furthermore, in the  $\beta$ -series the 2. 4. 6-tribromophenyl glucoside<sup>17</sup> exhibits a lower levorotation than the non-brominated substance. According to a private communication of Dr. A. Kunz, the substitution of chlorine, bromine, and iodine in the benzene ring of the saligenin- $\beta$ -glucoside (salicin) causes a gradual decrease of levorotation and finally a change into dextrorotation.

4. Of special importance is the case of fructose. Hudson<sup>18</sup> has found that the  $\alpha$ - and  $\beta$ -forms of the pentacetate of this sugar are not interconvertible by the use of zinc chloride and further that fructose tetracetate does not exhibit mutarotation. In other words, the groups attached to carbon atom (2) show little tendency to change of position. This condition should be favor-

<sup>13</sup> Hudson, C. S., *J. Am. Chem. Soc.*, 1924, xli, 462.

<sup>14</sup> Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1925, lxiii, 85. Levene, P. A., *Chem. Rev.*, 1925, ii, 179.

<sup>15</sup> Clough, G. W., *J. Chem. Soc.*, 1918, cxiii, 526.

<sup>16</sup> Wrede, F., and Zimmermann, W., *Z. physiol. Chem.*, 1925, cxi, 65.

<sup>17</sup> Fischer, E., and Strauss, H., *Ber. chem. Ges.*, 1912, xlv, 2467.

<sup>18</sup> Hudson, C. S., *J. Am. Chem. Soc.*, 1915, xxxvii, 2736.

able for the preparation of two halogen derivatives. These were actually obtained.

The rotations of the pentacetates and of the chloro derivatives and nitro derivatives are given in Table III.

We are inclined to believe that Fischer and Armstrong<sup>19</sup> once

TABLE III.  
*Rotations and Melting Points of Acetohalogen Hexoses.*

(1)	$[\alpha]_D$ (2)	m.p. (3)	(4)	$[\alpha]_D$ (5)	m.p. (6)	Values of Columns 5-2. (7)
$\alpha$ -Fructose pentace- tate.	+34.7°	70°	$\alpha$ -Aceto- chloro- fructose.	-160.9°	83°	-195.5°
$\beta$ -Fructose pentace- tate.	-120.5°	108-109°	$\beta$ -Aceto- chloro- fructose.	+45.3°	108°	+166°
$\alpha$ -Glucose pentace- tate.	+101.6°	112-113°	$\alpha$ -Aceto- chloro- glucose.		63°	
			$\alpha$ -Aceto- bromo- glucose.		79-80°	
			$\alpha$ -Aceto- nitro- glucose.*	+1.5°	92°	-100°
$\beta$ -Glucose pentace- tate.	+3.8°	134°	$\beta$ -Aceto- chloro- glucose.	+165°	73°	+161°
			$\beta$ -Aceto- bromo- glucose.	+199.2°	88°	+195°
			$\beta$ -Aceto- nitro- glucose.	+149°	150°	+145°

\* Skraup, Z. H., and Kremann, R., *Monatsh. Chem.*, 1901, xxii, 375.

actually prepared the  $\alpha$ -form of acetochloro- and bromoglucose. The fact that subsequently only the  $\beta$ -form crystallized is not without precedent in sugar chemistry. In our own laboratory  $\beta$ -mannose on recrystallization always is transformed into the

<sup>19</sup> Fischer, E., and Armstrong, E. F., *Ber. chem. Ges.*, 1901, xxxiv, 2885; 1911, xlv, 1898.



$\alpha$ -form, whereas in other laboratories the  $\alpha$ -form never could be prepared. Again chondrosamine when prepared for the first time crystallized in the  $\alpha$ -form and subsequently always in the  $\beta$ -form.

It is noteworthy that for all the chloro-, bromo-, and nitroacetyl monoses in those cases in which both forms are known, the  $\alpha$ -forms have a lower melting point than the  $\beta$ -forms. In this respect they behave similarly to the  $\alpha$ - and  $\beta$ -pentacetates to which reference was made above.

#### CONCLUSIONS.

1. On the basis of the values of the optical rotation of carbon atom (1) of the pentacetates of glucose, galactose, and mannose it may be concluded that the ordinary  $\alpha$ - and  $\beta$ -pentacetates of galactose and of mannose possess an amylenoxidic structure.

2. It is further concluded that the isomeric (1)-methyl and (1)-ethyltetraacetyl mannoses possess different ring structures. Those derived from methyl or ethylmannoside possess the  $<1, 5>$  and those derived from (1)-bromotetraacetyl mannose possess a different ring structure.

3. It is pointed out that the "lactal" structure of monoses causes the allocation of the acetyl group attached to the carbon atom (1) and of the carbon atoms extending beyond the ring, to be orientated either on the same side or on different sides of the plane of the ring. One form may be regarded a *cis* and the other a *trans* structure. When the ring is to the right (in the *d*-series) the  $\alpha$ -forms (hydroxyl of carbon atom (1) on the same side as that of carbon atom (5)) should be the *trans* forms. When the ring is to the left the  $\beta$ -form has the *trans* configuration. This concept may in certain instances be helpful in deciding in favor of one or the other ring structures.

4. It was suggested that the common forms of (1)-halogen acetylated sugars may be regarded as the  $\beta$ -forms.

## ACETYL MONOSES. II.

BY P. A. LEVENE AND HARRY SOBOTKA.

(From the Laboratories of The Rockefeller Institute for Medical Research,  
New York.)

(Received for publication, January 23, 1926.)

The present paper contains a report on the preparation of a crystalline (1)-bromotetracetyl mannose (I) which served as starting point for the preparation of a (1)-methyltetracetyl mannose (II), m.p. = 105°C. and  $[\alpha]_D = -24.7^\circ$ . This substance was the same as the  $\gamma$ -form of Dale and on saponification with alkali led to a (1)-methylmonoacetyl mannose (III).

A second (1)-methyltetracetyl mannose (IV) was prepared from the  $\alpha$ -methyl mannoside and melted at 62–63° and had  $[\alpha]_D = +49^\circ$ . From this on saponification with alkali the original  $\alpha$ -methyl mannoside was obtained, this form being identical with the  $\alpha$ -form of Dale.

The case of acetylated ethyl mannoside presented special interest. Here also three forms were obtained. Ethyl mannoside was obtained only as a syrupy product by the action of 99.8 per cent ethyl alcohol containing 0.25 per cent of hydrogen chloride on mannose. On acetylation with acetic anhydride and sodium acetate, a small amount of a crystalline product was obtained and the larger part as a syrup. The crystalline product (V) had m.p. = 110–113°C. and  $[\alpha]_D = +1.8^\circ$  ( $\text{CHCl}_3$ ). The syrup (VI) had a specific rotation of +42.5°. Both these forms on saponification lost four acetyl groups. A third form (VII) was derived from the (1)-bromotetracetyl mannose, with a m.p. = 81–82°C. and  $[\alpha]_D = -28^\circ$ . On saponification, it behaved in a manner analogous to the methyl derivative prepared from the same source.

Thus it is evident that the ring structure of the first and second forms (V and VI) is different from that of the third form (VII). Hence, the first and second may be regarded as  $\beta$ - and  $\alpha$ -forms of one and the same ring isomer.

The third form of the ethyl and the levorotatory form (II) of the (1)-methylacetyl derivative apparently possess the same ring structure. Bromotetracetyl mannose has hitherto been obtained only in the form of a syrup. From such material Dale<sup>1</sup> prepared a methyltetracetyl mannose which resembled the  $\gamma$ -methyltriacetylrrhamnose of Fischer, Bergmann, and Rabe.<sup>2</sup> Dale further converted the  $\delta$ -form into an isomeric form, which was not a mutamer of either the  $\alpha$ - or  $\gamma$ -form. For these derivatives no mutameric forms are known.

The first and the second forms of the (1)-ethyltetracetates have a great significance in many respects. First they permit the prediction of the approximate specific rotation of the  $\beta$ -form of (1)-methyltetracetyl mannoside. As a rule, the magnitudes of rotation of the ethyl and methyl glucosides differ by only a few

TABLE I.  
*Differences of Molecular Rotations (Times 100) of  $\alpha$ - and  $\beta$ -Forms.*

	Glucose.	Mannose.
Free sugar.....	16,900	9,400
(1)-Methyl.....	37,400	
(1)-Ethyl.....	38,300	22,500
(1)-Methyl diacetone.....		21,200
Pentacetate.....	38,100	32,200

degrees, the ethyl having the higher rotation by about 5°. Thus the rotation of the  $\beta$ -methyltetracetyl form should be about -3°.

In Table I are given the values of the differences in the molecular rotations of  $\alpha$ - and  $\beta$ -mannose and of those mannosides which are known in two mutameric forms.

From this table it is seen that the differences of the rotations of the  $\alpha$ - and  $\beta$ -forms of mannose and of substituted and non-substituted mannosides amount to about 50 per cent of the corresponding differences for glucose derivatives. On the other hand, the value for the pentacetates of mannose is 80 per cent of the corresponding figure for glucose. The theoretical significance of these differences in the rotations was discussed in the previous paper.

<sup>1</sup> Dale, J. K., *J. Am. Chem. Soc.*, 1924, xlii, 1046.

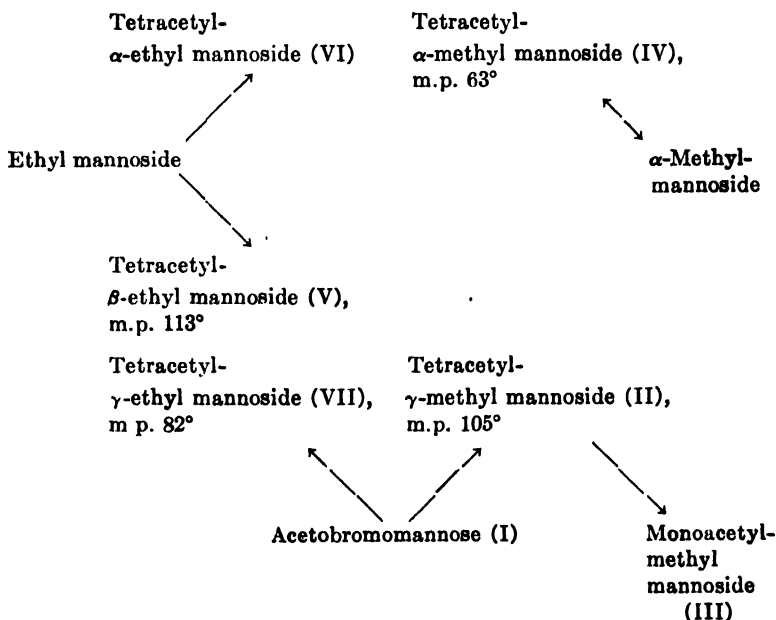
<sup>2</sup> Fischer, E., Bergmann, M., and Rabe, A., *Ber. chem. Ges.*, 1920, liii, 2362.

The present paper contains also a report on the two (1)-methyl-tetracetyl galactoses. One was prepared from the  $\alpha$ -methyl galactoside and was obtained in the form of a syrup with  $[\alpha]_D = +69.3^\circ$ .

The  $\beta$ -form was prepared from the (1)-bromotetracetyl galactose. The same substance had been prepared by Koenigs and Knorr,<sup>3</sup> from (1)-nitrotetracetyl galactose. It had a m.p. of  $94^\circ$  and  $[\alpha]_D = -10.5^\circ$  (in  $\text{CHCl}_3$ ). Each form on alkaline hydrolysis liberated four acetyl groups and hence none of them belonged to the group which firmly holds an acetyl radicle.

We also wish to record our failure to obtain the  $\beta$ -methyl mannoside starting from  $\beta$ -methyl diacetone mannose.

*Diagram of the Correlation of Acetylated Mannoses.*



**EXPERIMENTAL PART.**

*Acetobromomannose* (I) has been prepared from a sample of pure crystalline mannose  $\beta$ -pentacetate having a melting point of  $114^\circ$  and a specific rotation of  $-22.9^\circ$  in chloroform. 20 gm. of

<sup>3</sup> Koenigs, W., and Knorr, E., *Ber. chem. Ges.*, 1901, xxxiv, 957, 979.

the pentacetate were dissolved in 100 cc. of hydrobromic acid-glacial acetic acid. After 2 hours standing at room temperature, the solution was poured into three times its volume of chloroform. After thoroughly washing with ice water the chloroform solution was dried over calcium chloride and concentrated at low temperature under diminished pressure. A thick syrup was obtained. The yield was from 80 to 85 per cent of the weight of the pentacetate. After 2 days standing in a high vacuum over phosphorus pentoxide and soda lime nearly rectangular rhombic platelets crystallized, but could not be separated from the surrounding viscous decomposable syrup. The rotation in chloroform was:

$$[\alpha]_D = \frac{+10.60^\circ \times 100}{2 \times 4.340} = +122.1^\circ.$$

0.1014 gm. substance: 2.45 cc. 0.1 N AgNO<sub>3</sub> (Volhard).

C<sub>14</sub>H<sub>19</sub>O<sub>8</sub>Br (411.07). Calculated. Br 19.46.

Found. " 19.31.

From a syrupy mixture of the  $\alpha$ - and  $\beta$ -pentacetates similar yields of acetobromomannose could be obtained. It was necessary, however, to prolong the action of the hydrobromic acid for 4 hours. Depending upon the remaining traces of chloroform, specific rotations from  $+110^\circ$  to  $+120^\circ$  were thus obtained.

*Tetracetylmethyl mannoside*<sup>4</sup> (II) is formed by shaking 15 gm. of freshly prepared acetobromomannose with 20 gm. of dry silver carbonate in 200 cc. of absolute methyl alcohol for 6 to 12 hours. After a sample of the liquid had been found to be free of bromine, the whole solution was separated from the silver salts. In a few instances the presence of a small amount of silver acetate interfered with the filtration. A few cc. of methyl alcohol containing hydrogen sulfide had to be added and the silver sulfide removed. By concentration a crystalline mass was obtained. The needles can be recrystallized from a very small amount of methyl alcohol, a fairly small volume of 50 per cent methyl alcohol, or from benzene or from warm water. In the latter case care should be taken lest the temperature exceed  $50^\circ$  even though the solution be neutral toward litmus; otherwise, a considerable amount of an

<sup>4</sup> Brauns briefly mentions that he obtained only a syrup when trying to introduce a methyl group into the crystalline acetobromomannose (Brauns, D. H., *J. Am. Chem. Soc.*, 1922, xlv, 401).

isomer might be formed and prevent crystallization of the whole syrup. The yield was 12 gm., the melting point 105°.

0.1053 gm. substance: 0.1920 gm. CO<sub>2</sub> and 0.0572 gm. H<sub>2</sub>O.

C<sub>18</sub>H<sub>32</sub>O<sub>10</sub> (362.18). Calculated. C 49.72, H 6.08.

Found. " 49.72, " 6.07.

0.1294 gm. substance: 11.41 cc. 0.1 N NaOH.

Acetyl groups by alkaline hydrolysis: 3.19.

$$[\alpha]_D^{25} = \frac{-1.00^\circ \times 100}{2 \times 1.5} = -33.4^\circ \text{ in methyl alcohol.}$$

$$[\alpha]_D^{25} = \frac{-3.10^\circ \times 100}{2 \times 5} = -31.0^\circ \text{ in chloroform.}$$

$$[\alpha]_D^{25} = \frac{-2.22^\circ \times 100}{2 \times 1.5} = -74.0^\circ \text{ in benzene.}$$

When using ethyl instead of methyl alcohol a levorotatory *tetracetyethyl mannoside* (VII) is formed by an analogous procedure. It is recrystallized advantageously from the hundredfold amount of petroleic ether (b.p. 40–60°) in hard needles melting at 81–82°.

0.1046 gm. substance: 0.1960 gm. CO<sub>2</sub> and 0.0602 gm. H<sub>2</sub>O.

C<sub>18</sub>H<sub>34</sub>O<sub>10</sub> (376.19). Calculated. C 51.06, H 6.38.

Found. " 51.09, " 6.44.

0.1388 gm. substance: 11.64 cc. 0.1 N NaOH.

Acetyl groups found: 3.13.

$$[\alpha]_D = \frac{-0.56^\circ \times 10}{1 \times 0.1205} = -46.5^\circ \text{ in ethyl alcohol.}$$

$$[\alpha]_D = \frac{-0.48^\circ \times 10}{1 \times 0.174} = -27.6^\circ \text{ in chloroform.}$$

$$[\alpha]_D = \frac{-3.85^\circ \times 10}{1 \times 0.480} = -80.2^\circ \text{ in benzene.}$$

$$[\alpha]_D = \frac{-1.80^\circ \times 10}{1 \times 0.4725} = -38.1^\circ \text{ in ether.}$$

By the usual method we prepared the crystalline acetobromogalactose from the  $\beta$ -pentacetate. From the bromo compound tetracetyl- $\beta$ -methyl galactoside was prepared. After removal of the silver salts and of the methyl alcohol, the resulting syrup

was allowed to stand. After a few days it was wholly converted into a mass of prismatic crystals, some as long as 15 mm. The substance melted at 94°C., and was identical with that obtained by Koenigs and Knorr from acetonegalactose.

0.1012 gm. substance: 0.1830 gm. CO<sub>2</sub> and 0.0562 gm. H<sub>2</sub>O.

C<sub>18</sub>H<sub>22</sub>O<sub>10</sub>. Calculated. C 49.72, H 6.08.

Found. " 49.31, " 6.21.

0.1003 gm. substance: 11.50 cc. 0.1 N NaOH.

Acetyl groups found: 4.15.

$$[\alpha]_D = \frac{-0.24^\circ \times 10}{1 \times 0.532} = -4.5^\circ \text{ in methyl alcohol.}$$

$$[\alpha]_D = \frac{-0.53^\circ \times 10}{1 \times 0.5045} = -10.5^\circ \text{ in chloroform.}$$

$$[\alpha]_D = \frac{-1.24^\circ \times 10}{1 \times 0.492} = -25.2^\circ \text{ in benzene.}$$

*Tetracetyl- $\alpha$ -Methyl Mannoside (IV).*—20 gm. of  $\alpha$ -methyl mannoside and 10 gm. of fused anhydrous sodium acetate were heated for 1 hour in 100 gm. of acetic anhydride on the steam bath. The residue after removal *in vacuo* of unchanged anhydride was extracted with ethyl alcohol. 15 gm. of crystals melting at 62–63° were isolated.

0.1092 gm. substance: 0.1998 gm. CO<sub>2</sub> and 0.0598 gm. H<sub>2</sub>O.

C<sub>18</sub>H<sub>22</sub>O<sub>10</sub>. Calculated. C 49.72, H 6.08.

Found. " 49.80, " 6.12.

0.1668 gm. substance: 18.83 cc. 0.1 N NaOH.

Acetyl groups found: 4.03.

$$[\alpha]_D = \frac{+2.33^\circ \times 10}{1 \times 0.3575} = +65.2^\circ \text{ in methyl alcohol.}$$

$$[\alpha]_D = \frac{+2.47^\circ \times 10}{1 \times 0.5040} = +49.0^\circ \text{ in chloroform.}$$

$$[\alpha]_D = \frac{+3.00^\circ \times 10}{1 \times 0.5015} = +59.8^\circ \text{ in benzene.}$$

The analogous acetylation of  $\alpha$ -methyl galactoside led to the formation of *tetracetyl- $\alpha$ -methyl galactoside*. It could be distilled under a pressure of 0.8 mm. of mercury sharply at 190° as a colorless syrup. As yet it has not been crystallized.

0.1072 gm. substance: 0.1960 gm. CO<sub>2</sub> and 0.0582 gm. H<sub>2</sub>O.

C<sub>15</sub>H<sub>22</sub>O<sub>10</sub>. Calculated. C 49.72, H 6.08.

Found. " 49.85, " 6.07.

0.1015 gm. substance: 11.00 cc. 0.1 N NaOH.

Acetyl groups found: 3.92.

$$[\alpha]_D = \frac{+1.68^\circ \times 10}{1 \times 0.190} = +88.4^\circ \text{ in methyl alcohol.}$$

$$[\alpha]_D = \frac{+3.52^\circ \times 10}{1 \times 0.5088} = +69.3^\circ \text{ in chloroform.}$$

$$[\alpha]_D = \frac{+3.44^\circ \times 10}{1 \times 0.470} = +73.2^\circ \text{ in benzene.}$$

By the action of alcoholic hydrochloric acid upon mannose, as yet no crystalline ethyl mannoside has been obtained. The resulting syrup, which on account of its strong dextrorotation might be assumed to contain a predominating share of the  $\alpha$ -form, was acetylated in the usual way with acetic anhydride and sodium acetate. A syrup was obtained which analyzed for tetracetyl-ethyl mannoside.

C<sub>18</sub>H<sub>24</sub>O<sub>10</sub>. 0.1056 gm. substance: 0.1984 gm. CO<sub>2</sub> and 0.0614 gm. H<sub>2</sub>O.

Calculated. C 51.06, H 6.50.

Found. " 51.23, " 6.50.

By standing under petroleic ether and occasional scratching with a glass rod, flat prismatic needles began to crystallize (V). Since their amount did not increase after a few weeks, the syrup, after decantation of the petroleic ether, was taken up with thoroughly cooled ether. By centrifuging, 0.4 gm. of the crystals could be separated from 20 gm. of syrup.

After recrystallization from chloroform their corrected melting point was found to be 110–113°. The specific rotation was slightly positive in chloroform.

$$[\alpha]_D = \frac{+0.09^\circ \times 2.5}{1 \times 0.1222} = +1.8^\circ,$$

and negative in benzene

$$[\alpha]_D = \frac{-0.045^\circ \times 2.5}{1 \times 0.0747} = -1.5^\circ.$$



0.0774 gm. substance: 0.1463 gm. CO<sub>2</sub> and 0.0474 gm. H<sub>2</sub>O.

C<sub>16</sub>H<sub>24</sub>O<sub>10</sub>. Calculated. C 51.06, H 6.50.

Found. " 51.54, " 6.85.

0.0977 gm. substance: 10.63 cc. 0.1 N NaOH.

Acetyl groups found: 4.09.

After removal of the ether, the remaining syrup (VI) gave the following data:

0.1310 gm. substance: 14.15 cc. 0.1 N NaOH.

Acetyl groups found: 4.07.

$$[\alpha]_D = \frac{+2.26^\circ \times 10}{1 \times 0.5325} = +42.5^\circ \text{ in chloroform.}$$

$$[\alpha]_D = \frac{+1.58^\circ \times 10}{1 \times 0.347} = +45.6^\circ \text{ in benzene.}$$

The titrated solutions containing the products of alkaline hydrolysis were made up to a definite volume (30 and 65 cc.).

The specific rotations calculated for the resulting deacetylated ethyl mannosides were:

$$[\alpha]_D = \frac{+0.03^\circ \times 30}{2 \times 0.054} = +8.3^\circ \text{ from (V).}$$

$$[\alpha]_D = \frac{+0.26^\circ \times 65}{2 \times 0.074} = +114^\circ \text{ from (VI).}$$

These figures may approach the values for  $\beta$ - and  $\alpha$ -ethyl mannoside respectively.

*Hydrolysis of Tetracetyl- $\alpha$ -Methyl Mannoside.*—Dry ammonia gas was passed for 1 hour through a 10 per cent solution of tetracetyl- $\alpha$ -methyl mannoside. After evaporation of the ammonia and the methyl alcohol,  $\alpha$ -methyl mannoside was recovered which after recrystallization from methyl alcohol showed the exact melting point of 194° and the specific rotation:

$$[\alpha]_D = \frac{+1.04^\circ \times 10}{1 \times 0.1284} = +80.2^\circ \text{ in methyl alcohol.}$$

*Hydrolysis of tetracetyl- $\gamma$ -methyl mannoside by ammonia.* The same scheme was applied to the levorotatory tetracetylmethyl mannoside. By extraction with acetone, it was separated from the ammonium acetate; the viscous substance from the acetone

solution showed a very slight levorotation corresponding to a specific rotation of less than  $-2^\circ$ .

*Monoacetylmethyl Mannoside (III)*.—10 gm. of the levorotatory tetracetylmethyl mannoside were boiled with a double amount of crystalline barium hydroxide for 10 minutes in 750 cc. of water. The excess of barium was removed by passing in carbon dioxide and the concentrated residue was extracted with ethyl alcohol. The resultant solution was again concentrated; after repeating this procedure several times the substance was found to be free from barium acetate. In high vacuum a dry hygroscopic mass was obtained, which analyzed correctly for a monoacetylmethyl mannoside.

0.0928 gm. substance: 0.1554 gm.  $\text{CO}_2$  and 0.0580 gm.  $\text{H}_2\text{O}$ .  
 $\text{C}_9\text{H}_{16}\text{O}_7$  (236.13). Calculated. C 45.74, H 6.83.  
Found. " 45.66, " 6.99.



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**PROCEEDINGS OF THE AMERICAN SOCIETY OF  
BIOLOGICAL CHEMISTS.**

**TWENTIETH ANNUAL MEETING.**

**Cleveland, Ohio, December 28-30, 1925.**



## **CAN OTHER IMIDAZOLES REPLACE HISTIDINE IN THE DIET FOR PURPOSES OF GROWTH?**

By GERALD J. COX AND WILLIAM C. ROSE.

*(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.)*

Studies have been made of the influence upon growth of synthetic compounds containing the imidazole ring. For this purpose the substances in question were added to a basal diet in which the nitrogen was supplied in the form of completely hydrolyzed casein from which histidine had been removed by precipitation with silver sulfate and barium hydroxide. No influence upon the growth of rats was observed following the inclusion in the ration of imidazole, 4-methyl imidazole, 4-hydroxymethyl imidazole, 4-imidazole formaldehyde, 4-imidazole carboxylic acid, 4-imidazole acetic acid, 4-imidazole propionic acid, or 4-imidazole acrylic (urocanic) acid. The inclusion in the diet of one or two equivalents of either of the above substances entirely failed to inhibit the loss in weight occasioned by the deficiency of histidine.

On the other hand, the addition of *dl*-4-imidazole lactic acid to the histidine-deficient diet caused an immediate resumption of growth, but at a rate slightly slower than that induced by the equivalent quantity of histidine. The above results constitute the first successful attempt to replace an "indispensable" amino acid of the diet by a non-amino compound.

## **THE CHEMICAL CONFIGURATION OF THYROXIN AND ITS MODE OF ACTION IN THE TISSUES.**

By EDWARD C. KENDALL.

*(From the Section of Biochemistry, Mayo Foundation, Rochester, Minnesota.)*

The recent work of Hicks at Cambridge, England, has demonstrated by means of the absorption of the ultra-violet spectrum the presence of the indole nucleus in thyroxin. Furthermore, he has shown that the pyrrolidone ring is open or closed under conditions

similar to those in which I have shown it to be open or closed by chemical tests. The only remaining problem, therefore, with the configuration of thyroxin lies in the position of the 3 atoms of iodine and the 2 atoms of hydrogen. By synthesis, we have eliminated the possibility of one of the double bonds being in the pyrrolidone ring and also by synthesis, we have proved the stability of the halogens in the benzene ring when they are in positions 4, 5, and 6.

The difficult problem in the synthesis of these compounds has been to add the third halogen. This has recently been accomplished and 3 atoms of bromine have been added to the benzene ring in positions 4, 5, 6. When the 3 atoms of bromine are added to the unreduced benzene ring in positions 4, 5, and 6, a compound is produced whose chemical properties are not similar to those of thyroxin. Attempts were therefore made to reduce the benzene ring and add 2 atoms of hydrogen. This cannot be brought about by reduction but further investigation showed the benzene ring is sensitive to oxidation. The tribromo compound with bromine atoms in positions 4, 5, 6 will add 2 more atoms of bromine on positions 5 and 6, forming a pentabromo derivative. This substance is surprisingly stable and it is reduced with difficulty to a tribromo 4, 5, 6 leaving 2 atoms of hydrogen, one on 5 and one on 6. This substance has the formula assigned to thyroxin except there are 3 atoms of bromine in the positions occupied by the 3 atoms of iodine. The chemical properties of this compound are strikingly similar to those of thyroxin. It is stable to reduction and to strong alkali, exists with the pyrrolidone ring open or closed, and gives the unique color reaction with nitrous acid and an alkali.

The physical and chemical properties of the tribromo thyroxin confirm as strongly as anything can the correctness of the formula assigned to thyroxin.

By means of a study of the oxidation-reduction potentials of a number of these synthetic compounds which are closely related to thyroxin, it can be shown that the hydrogen on No. 7 carbon is easily oxidized. Dibromindolephenol in the complete absence of oxygen is capable of oxidizing any of these derivatives. The fully reduced and fully oxidized forms have very little effect on the platinum electrode. The partially oxidized form however develops a strong reducing potential and indicates that there

exists an intermediate compound possibly possessing the properties of a free radical, which is the actively functioning form of this series of compounds. Further work has shown that epinephrine behaves in an entirely similar manner. Neither its fully reduced or fully oxidized form possesses the reactivity of the intermediate partially oxidized molecule. Also cysteine-cystine and oxidized and reduced glutathione are other members of this group of substances. The fully oxidized and reduced forms of these compounds are not in equilibrium with each other but they are in equilibrium with the partially oxidized or free radical forms of the substances. Thyroxin, epinephrine, and glutathione are members of a group of substances which furnish to the animal organism an active chemical grouping. They establish the intensity of the oxidation-reduction process occurring within the cells, and produce a poisoning effect maintaining the intensity of oxidation and reduction within narrow limits.

#### SOME PROPERTIES OF THE HORMONE OF THE LIQUOR FOLLICULI.

By J. O. RALLS, C. N. JORDAN, C. T. HEUSINKVELD, AND EDWARD A. DOISY.

*(From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis.)*

Liquor folliculi has been preserved with two volumes of 95 per cent alcohol for a period of  $1\frac{1}{2}$  years without suffering loss of potency. The stability of highly purified products seems to be remarkably less than that of crude products.

Our best method of preparation (to be published soon) yields a product of which approximately 0.04 mg. is one rat unit. The method is based merely upon the differential solubility of the hormone in various common organic solvents.

Data were presented upon the effect of hydrolysis, hydrogenation, acetylation, benzylation, and bromination upon the activity of the hormone. The percentages of carbon, hydrogen, nitrogen, and phosphorus were determined on our best preparation of which 0.04 of a mg. was one rat unit.



**NOTES ON THE CHEMISTRY AND PHYSIOLOGY OF THE ANTI-PRESSOR FRACTION FROM HEPATIC TISSUE.**

By A. A. JAMES, N. B. LAUGHTON, AND A. BRUCE MACALLUM.

(From the Departments of Biochemistry and Physiology, University of Western Ontario Medical School, London, Ontario, Canada.)

We have obtained from absolutely fresh liver of herbivores, omnivores, and carnivores a substance giving a sustained fall in blood pressure. The yield from herbivores does not seem so consistent.

The fresh washed liver is well frozen and thawed two or three times in a slightly acid medium. The proteins are removed by treatment with alcohol up to 75 per cent at the isoelectric point and the whole allowed to stand 24 hours. After removal of the alcohol the active principle can be precipitated by phosphotungstic acid or acetone. In the case of the former the phosphotungstates are decomposed with baryta in a water-acetone suspension, taken to dryness, purified, and finally shaken in aqueous solution with ether. The ether contains a water-soluble substance which causes a sustained fall in blood pressure, gives no biuret reaction, no indication of histamine with *p*-diazobenzene sulfonic acid, or crystals of choline with iodine.

Acetone precipitates from the extract a pressor substance of unknown constitution which can be removed by shaking with Lloyd's reagent in  $\frac{1}{2}$  per cent acetic acid.

In clinical hypertension the preparation has a relatively more profound effect than on the normal mammalian blood pressure.

The site of action is not on the capillaries as in the case of histamine. There is no peripheral dilatation. In the mesenteric blood vessels there can be seen venous engorgement with slowing of the blood stream in veins and arterioles. The capillaries show no dilatation.

**KETOSIS IN THE RAT.**

By ARTHUR H. SMITH AND HAROLD LEVINE.

(From the Laboratory of Physiological Chemistry, Yale University, New Haven.)

Normal growth from 30 to 180 gm. of body weight can be obtained in rats on a high fat diet containing 86 per cent of the

total calories as fat calories. The utilization of fat in these experiments ranged from 96 to 99 per cent.

In metabolism experiments, no essential difference in the urinary output of "acetone bodies" was evident whether the diet was composed largely of fat, protein, or carbohydrate or consisted of a balanced mixture of these dietary essentials. On changing suddenly from a high carbohydrate or high protein diet to a high fat régime, no change in the level of the "acetone bodies" excretion was noticeable. The quantity of "acetone bodies" excreted by the normal rat is independent of the nature of the diet and varies in most animals from 1 to 3 mg. (expressed as acetone) per day. Fasting produced no appreciable ketosis in the rat contrasted to the human species.

The results mentioned above seem to point to a type of fat-carbohydrate metabolism in the rat which is different from that in man. Rats on any of the above mentioned régimes put out a rather constant proportion of beta-hydroxybutyric acid (expressed as acetone); *i.e.*, approximately 70 per cent of the "total acetone bodies." When sodium bicarbonate constituted 12 per cent of a high fat diet, an immediate rise in the output of "acetone bodies" was observed. On changing back to the high fat diet without the alkali, the "acetone bodies" excreted promptly fell to the former low level.

#### THE CONDUCTIVITY OF ERYTHROCYTES TO ELECTRIC CURRENTS OF HIGH AND LOW FREQUENCY.

By J. F. McCLENDON.

*(From the Laboratory of Physiological Chemistry, University of Minnesota,  
Minneapolis.)*

Frequencies from 120 to 4000 cycles are obtained from a Vreeland oscillator 10 meters from the bridge, high frequency currents up to a million, from an electron tube oscillator with toroidal coils 5 meters from the bridge. One corner of the bridge is grounded. The bridge consists of a meter platinum wire with sliding contact connected to the ground by means of a wire dipping in a mercury trough, and two conductivity cells made exactly alike with gold plates 25 mm. in diameter and 10 mm. apart. Conductivity of the blood is compared with that of a KCl solution

of about the same conductivity. The reactance is balanced by means of decade toroidal coils connected in series with the conductivity cell. All parts of the system have an electrostatic shield placed at least 3 inches from the conductors, and the conductivity cells are placed in an air thermostat. A special device keeps the blood corpuscles stirred up. A curve was shown of the ratio of conductivity of serum to conductivity of blood at different corpuscle volumes up to 99 per cent. Corpuscle volumes are determined by means of a hematocrit mounted on the shaft of a Dumore high speed electric motor. The resistance of serum is the same at a thousand and million cycles within 1 per cent. The resistance of corpuscles at 1000 cycles is five to eight times as great as at a million cycles. Laking does not change resistance of corpuscles to a million cycles more than 7 per cent but increases that to 1000 cycles to equal that to a million (within 20 per cent). The resistance of sugar-washed corpuscles to 1000 cycles is 50 times as great as of serum, but to a million cycles is eight times as great. The curve of  $R_b/R_s$  against cell volume, at 1000 cycles approaches Clerk Maxwell's formula assuming the corpuscles are insulators, while that to a million cycles diverges greatly but approaches that calculated, assuming less corpuscle resistance. As the electrolyte is diluted, the electric double layer becomes thicker. The resistance of the corpuscle interior is about equal to that of a 0.01 N KCl solution.

#### THE PRECIPITATION OF UREASE BY LEAD ACETATE.

By JAMES B. SUMNER.

(From the Departments of Physiology and Biochemistry, Cornell University Medical College, Ithaca.)

Urease is entirely precipitated from solution by a slight excess of neutral lead acetate. If the precipitate is centrifuged and stirred with sodium sulfate solution, much of the protein, but no urease, is liberated. After washing four times with sodium sulfate, the urease can be largely recovered by extraction with oxalate-phosphate solution. This urease is not so pure, in respect to its protein content, as that prepared by the cooling process.<sup>1</sup> It

<sup>1</sup> Sumner, J. B., and Graham, V. A., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 504.

appears to contain a globulin different from canavalin, concanavalin A and concanavalin B. Upon dialyzing there is no precipitate. Dialysis against thousandth normal acetic acid causes the solution to become opalescent. The addition of sodium chloride now causes the precipitation of insoluble urease.

Insoluble urease contains no iron and is entirely free from pectase, which is present in the jack bean in large amount.

### STUDIES ON GLUTELINS.

By D. BREESE JONES AND FRANK A. CSONKA.

*(From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)*

It was noted in certain solubility studies that sodium chloride solution may have a denaturing effect on proteins. Therefore, it is possible that during the extraction of seeds and raw materials with saline solutions the albumins and globulins become more or less denatured, and are thus rendered partly insoluble, the denatured portion remaining in the extracted residue. Since glutelins are generally prepared from residues that have been exhaustively extracted with salt solutions, the application of alkali as a solvent for the glutelins will dissolve these denatured proteins, and consequently the glutelin preparation obtained on neutralization of the alkaline extract with acid will consist of a mixture of proteins and not of glutelin alone.

It was found that both wheat gliadin and wheat glutenin are precipitable from their alkaline solutions by addition of very small quantities of ammonium sulfate. We separated two glutenin fractions. The first fraction was obtained by making the alkaline solution 0.02 to 0.04 saturated with ammonium sulfate, and the second fraction precipitated at 0.1 to 0.15 of saturation. Both preparations had an isoelectric point of 6.45, which was identical with that found for gliadin. Work is under way for a further characterization of these two fractions.

The filtrate from the second fraction, on further addition of ammonium sulfate to 0.2 to 0.3 of saturation, yielded a relatively small quantity of another precipitate, having an isoelectric point of 5.45. Both the isoelectric point and degree of saturation with ammonium sulfate at which this fraction was precipitated come well within the range of these constants for globulins.

**ON BIOCHEMICAL COLOR REACTIONS WITH BENZIDINE, *p*-PHENYLENE DIAMINE, ETC.**

By W. MANSFIELD CLARK, BARNETT COHEN, AND H. D. GIBBS.

*(From the Division of Chemistry, Hygienic Laboratory, United States Public Health Service, Washington.)*

The electromotive conduct of tolidine, *p*-amino dimethyl aniline, and similar diamines of the phenylene diamine and benzidine series has permitted: (1) The determination of basic dissociation constants of the diamine (reductant). (2) The determination of basic dissociation constants of the diimide (oxidant). (3) The "normal" oxidation-reduction potential. (4) The degree of association of those species of oxidant and reductant which form the so called meriquinone or colored compound used in tests of oxidative action.

The quantitative data have provided an outline of the very complicated set of equilibria involved in these color tests, and have shown the impracticability of attempting to make benzidine and similar reagents useful in precise tests of oxidizing intensity. Tentatively, however, the data when combined with reports in the literature, concerning the ability of cell suspensions to oxidize these diamines, indicate a very low level of oxidizing intensity.

**THE OXIDATION AND REDUCTION POTENTIALS OF SOME COMPOUNDS CLOSELY RELATED TO THYROXIN.**

By JOHN M. ORT.

*(From the Section of Biochemistry, Mayo Foundation, Rochester, Minnesota.)*

The oxidation-reduction potentials and chemical activity of 2-oxodihydroindole-3-propionic acid and some of its halogen derivatives have been determined. The results show that in both the fully reduced and fully oxidized forms these substances have very little effect on a platinum electrode. When the reduced form is partially oxidized an intermediate compound is produced which has a marked reducing potential.

The chemical activity of these compounds closely parallels these findings. The reduced form of the above compounds reduces dibromindophenol slowly. After partial reduction of the dibromindophenol, the action becomes more rapid and continues to

the complete reduction of this dye. The addition of 2 atoms of bromine or of iodine to the indole nucleus of these compounds markedly increases the rate of reduction of dibromindophenol but does not change the intensity of the reducing power of the intermediate or active state of the molecule.

#### **DIFFERENTIATING REACTIONS FOR CYSTEINE, CYSTINE, AND GLUTATHIONE.**

By M. X. SULLIVAN.

*(From the Division of Chemistry, Hygienic Laboratory, United States Public Health Service, Washington.)*

Cysteine and reduced glutathione give a purple-red color with sodium nitroprusside and ammonium hydroxide. Cystine and oxidized glutathione do not. Addition of sodium cyanide makes cystine and oxidized glutathione give the purple nitroprusside reaction. Cysteine gives a red color with 1.2 naphthoquinone-4-sodium sulfonate and sodium sulfite in alkaline solution. Cystine does not, but will give the red color if treated with NaCN before addition of the naphthoquinone. Glutathione does not give the red color with the naphthoquinone, either with or without treatment with sodium cyanide. Hydrolyzed glutathione gives the naphthoquinone reaction as given by cystine. By proper treatment with these reagents, with and without use of sodium cyanide, cysteine, cystine, and glutathione can be determined quantitatively in pure solution and roughly quantitatively at present in mixtures of the four compounds, cysteine, cystine, reduced glutathione, and oxidized glutathione.

#### **A NEW ACID-FORMING ENZYME IN GASTRIC AND OTHER TISSUES, AND ITS POSSIBLE SIGNIFICANCE IN THE GASTRIC HYDROCHLORIC ACID MECHANISM.**

By MARTIN E. HANKE.

*(From the Laboratory of Physiological Chemistry, University of Chicago, Chicago.)*

A new theory has been developed for the chemical mechanism of the formation of hydrochloric acid in the gastric juice, which assumes essentially the hydrolysis of an alkyl chloride by a specific enzyme activity with the production of the corresponding alkyl

alcohol and hydrochloric acid. In testing this theory experimentally the following results have been obtained.

1. When fresh aqueous gastric tissue extract is incubated with any one of a number of chloride esters, acidity is produced, which is a direct function of the amount of tissue extract and the amount of chloride ester used.

2. The "chloride ester activity" is quite distinct from the ordinary gastric and other tissue lipases.

3. Using glycerol  $\alpha$ -dichlorohydrin,  $\text{ClCH}_2\text{CHOHCH}_2\text{Cl}$ , in  $\text{m}/20$  solution ( $\text{N}/10$  chloride) and a 10 per cent fresh gastric tissue extract, about one-tenth of an equivalent amount of acid is formed after incubating for 15 hours at  $37^\circ\text{C}$ ., *i.e.*  $\text{N}/100$  acid. Using glycerol dichlorohydrin as standard, other chloride esters may be arranged in the order of their relative activities, thus:

Glycerol dichlorohydrin.....	100
"    monochlorohydrin..	28
Monochloroacetic acid.....	28
Dichloroacetic    ".....	16
Trichloroacetic    ".....	0
Chloroform.....	0

That the activity is a function of the chemical nature of the chloride ester is evident. It seems particularly significant that the monochlorohydrin and monochloroacetic acid *always* give the same amount of activity, and that they are both the same type of chloride ester, *i.e.* a monochloro ester of a primary alcohol.

4. The distribution of the "chloroesterase" in different tissues of normal dogs was observed by incubating equal amounts of dried, ether-extracted, aqueous tissue extracts with dichlorohydrin. The numbers in the following list represent the relative amounts of acidity produced with the respective tissues: liver 120, fundus (stomach) 100, pancreas 63, duodenum 31, ileum 19, pylorus (stomach) 17, muscle (skeletal) 15, brain 7, blood 6, heart 6. The relatively high activity in liver and pancreas tissues may be correlated with the active glandular character of these tissues, and the fact that they are relatively very active with respect to other common enzymes, *e.g.* lipase and erepsin. With exception of these two tissues, the "chloroesterase" activity in fundic stomach tissue is distinctly greater than it is in any other tissue so far studied. There is none of this activity in commercial pepsin.

5. In no case has it been possible to demonstrate a production of chloride ion corresponding in amount with the acidity developed, although this has been looked for under a variety of conditions by different methods. The acidity production therefore does not seem to be due to the formation of hydrochloric acid, and its possible significance in the normal gastric hydrochloric acid mechanism is rendered very questionable.

#### DISCUSSION.

The fact that it has not been possible to demonstrate chloride production along with the acidity production makes it appear unlikely what this "chloroesterase" is concerned with the normal gastric hydrochloric acid mechanism. On the other hand, there are two outstanding facts which lend weight to the probable significance of the enzyme in this connection. The amount of activity is always greater in the fundic than in the pyloric portion of the stomach, and greater in gastric than it is in any other tissue except liver. The activity is certainly a function of the presence of the chloride ester, and more specifically, the amount of activity is a function of the chemical nature of the organic chloride. We have thus far been unable to develop a plausible theory for the mechanism of the action of the chloride ester, aside from hydrochloric acid production, and we believe this phase of the question merits further investigation. Possibly the failure to show chloride ion production lies in the methods which have been used.

#### STUDIES ON THE PURIFICATION OF PEPSIN.

BY T. L. McMEEKIN AND F. C. KOCH.

*(From the Department of Physiological Chemistry, University of Chicago, Chicago.)*

Two quantitative methods for pepsin were devised based upon the rate of solution of properly prepared coagulated egg white. This rate of solution was determined in parallel by means of micro Kjeldahl estimations and refractive index readings. Remarkably consistent results were obtained by both methods and both were very satisfactorily applied upon unknown standard solutions of pepsin containing 0.03 to 8.9 mg. of pepsin.



In the purification studies advantage was taken of the specific absorption of pepsin upon properly treated coagulated egg white. The optimum conditions for completeness and greatest quantity of absorption of pepsin were found to be at a pH of 3 at room temperature. The pepsin can again be recovered from the egg white combination by treatment with dilute  $\text{NaHCO}_3$  solution at a pH of 7.2 but even here there is considerable destruction of pepsin. By means of a 0.2 per cent disodium hydrogen phosphate solution 90 to 96 per cent recovery was shown. The pepsin thus absorbed and released was at least eight times as active as the original 1:3000 U.S.P. preparation started with. It was further purified by precipitation by half saturation with ammonium sulfate at a definite pH and then dialyzed. The ammonium sulfate-free material was evaporated to dryness *in vacuo* over phosphorus pentoxide. The product obtained was 16.9 to 17.8 times as active as the 1:3000 U.S.P. product. It is protein in nature, but not of glycoprotein or nucleoprotein character.

#### PEPSIN AND THE AUTOLYTIC PROTEASE.

By HARRY BAERNSTEIN AND H. C. BRADLEY.

(From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

Pepsin and the primary protease of tissues are so similar as to be easily confused. The older literature on the distribution of pepsin may therefore be erroneous and in need of revision. Potentiometer control of H ion concentrations is often impracticable in field experiments. By selecting acids of lower dissociation constants than HCl it is possible to set up digests whose pH values may be predicted with sufficient accuracy to permit the complete inhibition of the tissue protease while allowing pepsin to function catalytically. Fairly strong oxalic acid solutions are adapted to this purpose and by their use we are reinvestigating the distribution of pepsin. By means of it the stomachs of the herbivorous carp and the carnivorous cod are found devoid of pepsin, while the stomachs of the dogfish and mackerel shark are found to contain it.

**A MODIFIED METHOD FOR THE DETERMINATION OF HIPPURIC ACID.**

BY WENDELL H. GRIFFITH.

*(From the Department of Biological Chemistry, St. Louis University School of Medicine, St. Louis.)*

Experimental studies on the synthesis of hippuric acid in the animal organism frequently necessitate the determination of hippuric acid in urines which may contain free benzoic acid and benzoylglycuronic acid in addition to hippuric acid. The methods of Folin and Flanders and of Kingsbury and Swanson cannot be used for the determination of hippuric acid in such urines because these procedures determine total benzoic acid rather than that fraction present as benzoylglycine. The methods of Dakin and of Snapper and Laqueur, which may be used for the determination of hippuric acid, are long and tedious.

The following procedure has been found to be more satisfactory than any of the above methods. 10 cc. of protein-free urine, acidified with HCl, are extracted with ether for 1 hour in a continuous extraction apparatus. The only nitrogenous substances present in the ether residue in significant amount are hippuric acid and urea. Complete decomposition of the urea is effected by shaking the residue with bromine water and then with NaOBr solution. Hippuric acid nitrogen is determined by the Kjeldahl method. Although hippuric acid is not readily soluble in ether, as much as 250 mg. can be extracted from 10 cc. of urine in 1 hour. The entire determination is carried out in the same 500 cc. Kjeldahl flask. This procedure gives accurate results and greatly reduces the number of laboratory manipulations which are necessary in the methods of Dakin and of Snapper and Laqueur.

**THE ESTIMATION OF THE TOTAL PHOSPHORUS AND LIPOID PHOSPHORUS OF THE BLOOD.**

BY JOSEPH H. ROE.

*(From the Department of Biochemistry, George Washington Medical School, Washington.)*

The molybdic oxide colorimetric method introduced by Taylor and Miller, modified by Bell and Doisy, Briggs, and Benedict and

This is accurate when: (1) the blood is ashed at temperatures below 200°C.; (2) coloration is produced in acid concentrations ranging from 0.9 N to 1.9 N  $\text{H}_2\text{SO}_4$ ; (3)  $\frac{1}{2}$  to 1 cc. of 5 per cent ammonium molybdate and 1 cc. of 1 per cent hydroquinone in 20 per cent sodium sulfite are used; (4) the phosphomolybdic acid mixture is boiled on a water bath for 15 minutes.

### A NEW METHOD FOR POTASSIUM.

By CYRUS H. FISKE AND GEORGES LITARCZEK.

*(From the Department of Biological Chemistry, Harvard Medical School, Boston.)*

The shortcomings of the cobalti-nitrite method, especially for the accurate analysis of urine, have led us to search for some other precipitating agent suitable for use with comparatively small quantities of potassium. For the analysis of potassium salt solutions, in the presence of a limited amount of sodium, the acid tartrate has much to recommend it. It can be precipitated quantitatively by means of alcoholic solutions of tartaric acid, and is susceptible to accurate titration with standard alkali. The tartrate method is, however, not specific. We have sought, therefore, to combine it in some way with the cobalti-nitrite precipitation, for the latter is an ideal means of isolating the potassium in fairly concentrated form, free from most other constituents of urine, blood, and other biological products.

The disadvantage of using a double precipitation method is more than offset by the fact that ashing of the urine, etc., is dispensed with. The same result is attained, with considerably less trouble, by ashing the cobalti-nitrite precipitate itself—it need not even be removed from the centrifuge tube in which it is thrown down. Decomposing the cobalti-nitrite with hot acid, as a preliminary to precipitation as the acid tartrate, would not do; the solution would contain ammonium salts and possibly organic matter, and cobalt (in solution) also interferes. By ashing, every interfering substance is either destroyed or driven off. The small amount of sodium which the precipitate contains is not thrown down by tartaric acid; and the cobalt is converted to the oxide, which is so little soluble that its presence does no harm, even if it is not removed at any time.

Ignition of the cobalti-nitrite precipitate leaves a residue containing the potassium in the form of nitrite, mixed with cobaltous oxide and some sodium nitrite. From this mixture, taken up in a small volume of water, alcoholic tartaric acid precipitates only the potassium, and the separation is complete in a few minutes. The acid potassium tartrate is then filtered off, washed with alcohol, and titrated with 0.02 N sodium hydroxide.

With this method, from 0.4 to 4 mg. of potassium may be determined within about 0.01 mg.

#### THE PHYSIOLOGICAL SIGNIFICANCE OF BACTERIAL DEAMINATION.

By HORACE B. SPEAKMAN.

*(From the Department of Zymology, University of Toronto, Toronto, Canada.)*

The process of deamination has been studied during a normal fermentation of carbohydrate by a bacillus. By chemical methods the products have been identified. The course of deamination has been correlated with vegetative growth and the primary and secondary oxidation of glucose.

Deamination is not associated with growth but with vigorous intercellular oxidation. The acid products are secreted by the cells, but the ammonia is utilized. Single amino acids and ammonium phosphate do not support growth, but they catalyze carbohydrate oxidation by resting cells. The specific dynamic action of amino acids is due to the catalytic effect of the liberated ammonia which passes through some unknown cycle within the cells.

#### STUDIES ON CHOLESTEROL.

#### THE RELATION OF THE SUPRARENALS AND THE SPLEEN TO CHOLESTEROL METABOLISM.

By F. S. RANGLES AND ARTHUR KNUDSON.

*(From the Department of Biological Chemistry, Albany Medical College, Albany.)*

It has been held that the suprarenal gland is in some way closely associated with the cholesterol metabolism, and that this gland is capable of synthesizing cholesterol. In order to throw some light

upon this question the adrenals were removed from about thirty rats. Most of these rats were placed on a cholesterol-free diet while the remainder were placed on the same diet with cholesterol added. After varying lengths of time the rats were killed and the cholesterol content of several tissues, particularly the blood, was studied. No marked departure from the normal cholesterol content could be noted in either series of rats.

The same experiment was tried, removing the spleens from rats, since this organ has been suggested as having a cholesterogenic function. In a series of twelve splenectomized rats, part on a cholesterol-free diet and part receiving cholesterol, no pronounced variation from the normal could be found in the cholesterol content of the blood.

#### THE VARIATIONS IN THE SUGAR CONTENT OF HUMAN URINE.

By FRANCIS B. KINGSBURY.

*(From the Biochemical Laboratories of the Metropolitan Life Insurance Company, New York.)*

53,599 urine specimens from applicants for life insurance and 12,239 specimens from employees of the company have been analyzed for sugar by the Benedict picric acid-acetone method modified for use in test-tubes. This method checked by the Folin-Berglund method gives values that average about 20 per cent higher than by the Folin-Berglund method with urines containing from 0.1 to 0.2 per cent sugar, but with urines containing from 0.2 to 0.5 per cent the average values in a series are the same by both methods.

Specimens from applicants for life insurance are sent to the home office from the whole territory east of the Rocky Mountains either because of abnormal findings by the examiners or because the medical division desires additional information. A certain number are sent in because the amount of insurance applied for is large, but since the rest are sent to the home office laboratory because there is doubt as to whether the applicant is a good risk the group as a whole is slightly less normal than the employee group with which they are compared. In this latter group the greater number have passed a physical examination on entering the employ of the company and the specimens obtained at this time and later at yearly intervals are the ones considered. The selection is not

complete, however, because there are included the records of some who failed to pass the physical examination. The larger part of the employee group are young women.

A survey of the data shows that with both groups there is a very distinct line of demarcation at the 0.2 per cent level of sugar, and that there is very little variation in either group month by month of the total percentages of specimens showing less than 0.2 per cent of sugar. The average percentage of applicants whose specimens contained less than 0.2 per cent of sugar is 91.5 and for the employees 96.5. From these facts it seems probable that what we call the physiological sugar must in nearly all cases be an amount less than 0.2 per cent. At levels above 0.2 per cent there are relatively more of the applicant group than of the employee group for the same sugar level. This is particularly noticeable as the diabetic level is approached, and is probably due in part at least to attempts on the part of the applicants with known diabetes to obtain insurance.

#### **SOME FACTORS INFLUENCING THE BASAL METABOLISM OF CHILDREN.**

BY GRACE MACLEOD AND MARY SWARTZ ROSE.

*(From the Department of Nutrition, Teachers College, Columbia University, New York.)*

Basal metabolism determinations have been made on a group of 13 children (6 girls and 7 boys) living in an institution in the country and on another group of 10 children (6 girls and 4 boys) living in a city orphanage, first early in December and then again in late March or early April. The Benedict Student Apparatus was used, being checked against the Benedict Portable Respiration Apparatus both before and after it had been removed from the college laboratory to the orphanages.

It was found that the basal metabolism of the city children without exception was lower in April than in December by amounts ranging from 3.1 to 16.9 per cent. Of the country children 6 gave lower values in March than in December, the differences ranging from 1.0 to 7.5 per cent; 4 had higher basal rates in March than in December, by amounts ranging from 2.0 to 8.1 per cent; and 1 gave the same result in March as in December. In the total of 17 comparisons the December figure was greater than the

March figure in 12 cases, less than it in 4 cases, and equal to it in 1 case. It was known that the city children were housed more during the winter months than the country children. Both groups of children lead very active outdoor lives during the summer months, the city children being sent to camps in the country for the whole summer. We seem to be dealing here with an effect like that observed by both Lusk and Rapport in their dogs when they were brought under laboratory conditions after a vacation period in the country. Another large group of city children is being studied this winter with the object of further investigating this effect.

In the country institution it was also possible to compare the basal metabolism figure obtained on a child before rising out of bed in the morning with that obtained on the same child when he or she had slept in another building and had risen, washed, dressed, walked to the building where the apparatus was located, and had a rest period of 25 to 30 minutes before the determination was made. 18 comparisons were obtained on 12 different children. In 7 cases the figure when the determination was made before the child had risen was less than when he or she had come in from another building, in 8 cases it was greater, and in 3 cases it was the same. The differences were well within the range of individual variations from day to day, from 1.1 to 6.8 per cent. The conclusion would seem to be that it is immaterial whether the determination of the basal metabolism of a child is made before rising, in the bed where he has spent the night, or after a rest period following the child's arrival at the laboratory in the morning after having spent the night in his own home. This is in agreement with the results of the study of college women made by Benedict and Crofts at about the same time that this work on children was being done.

#### THE INFLUENCE OF PROLONGED ADMINISTRATION OF EGG UPON THE HEMOGLOBIN CONTENT OF CHILDREN'S BLOOD.

By MARY SWARTZ ROSE.

*(From the Department of Nutrition and the Child Welfare Research Institute  
of Teachers College, Columbia University, New York.)*

As part of an extended study of the place of the egg in children's diets, hemoglobin determinations and erythrocyte counts were

made at intervals during 21 months (November, 1923, to July, 1925), on two groups of children between the ages of 2 and 6 years, the one group receiving a fairly good mixed diet from which eggs were excluded, the other being given in addition to a similar diet, one egg daily. The children were under constant supervision of a physician and a nutritionist, and the eggs were administered by the latter at the day nursery which all attended. The averages of the hemoglobin determinations on these children remaining in the group the whole time are as follows:

	Nov., 1923.	Mar., 1924.	July, 1924.	Nov., 1924.	Mar., 1925.	July, 1925.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Children receiving egg (10 cases).	82	83	89	79	86	84
“ “ no egg (9 cases).	88	84	77	72	80	80

The averages of the erythrocyte counts made in November, 1924, and July, 1925, on the whole number of children in each group at these times are as follows:

Children receiving egg (21 cases).		Difference	Children receiving no egg (17 cases).		Difference.
Nov., 1924.	July, 1925.		Nov., 1924.	July, 1925.	
		<i>per cent</i>			<i>per cent</i>
4,300,348	4,290,900	-2.2	4,215,320	3,974,140	-5.7

There was a positive, though not large difference in percentage of hemoglobin and number of erythrocytes in favor of the children receiving egg, and there was a close parallelism between these blood findings and the general physical improvement of the children.

#### SPECIFIC DYNAMIC ACTION FROM THE STANDPOINT OF THE SECOND AND THIRD LAWS OF THERMODYNAMICS.

By ELLIOTT T. ADAMS.

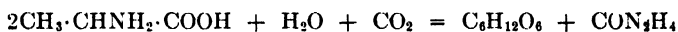
(From the Department of Biochemistry, Harvard Medical School, Boston.)

The theory has been advanced by Oppenheimer that the cells of the body must do chemical work in order to bring about some



of the metabolic transformations which follow the ingestion of food. This accounts, in his opinion, for the stimulating effect of some food substances upon metabolism. The results of free energy calculations are presented herewith which confirm this conception.

According to Lusk the following change in state takes place in the animal organism when alanine is ingested.



It is a consequence of the second law that if this change in state does not take place spontaneously, work will be required to make it take place. With the aid of the third law it is possible to show that approximately 60,000 small calories of work will be required to make the change in state take place ( $\Delta F = + 60,000$  calories). In order to do this work fuel must be burned in the organism. It is reasonable to suppose that the process will not take place in a reversible manner. Hence the fuel burned may be much larger than the minimum amount required to furnish the necessary work. This will explain, at least in part, the specific dynamic action of alanine. From this point of view the specific dynamic action of alanine is analogous (perhaps only in part) to the recovery process in muscle.

Lusk wrote the following equation to represent the metabolism of glycine.



The free energy increase for this change in state has been calculated with the aid of the third law and found to be approximately +196,000 calories. It is interesting to note that per mol of glucose formed the free energy increase is greater for the metabolism of glycine than for alanine. If Lusk's equation is physiologically correct this explains the relatively greater specific dynamic action of the former.

The calculations were made upon the assumption that the changes in state took place at 25°C. but otherwise under approximately the same conditions as are found in phlorhizinized dogs.

**DEPOSIT PROTEIN: THE EFFECT OF THYROXIN ON THE DEPOSIT  
PROTEIN AFTER REDUCTION OF THE NITROGEN EXCRE-  
TION TO A MINIMAL LEVEL BY A PROLONGED  
PROTEIN-FREE DIET.**

BY HARRY J. DEUEL, JR., KATHLEEN SANDIFORD, IRENE  
SANDIFORD, AND WALTER M. BOOTHBY.

*(From the Section of Clinical Metabolism, Mayo Foundation, Rochester,  
Minnesota.)*

That thyroxin administration causes an increased protein catabolism as evidenced by the urinary nitrogen excretion is well known<sup>2</sup> but whether such an effect is indicative of a toxic action on protoplasmic tissue or the result of an increased rate of destruction of deposit protein is undecided. In the present experiment an attempt was made to remove a considerable proportion of the deposit protein, at least the more labile fraction, and then to determine what effect thyroxin exerts on protein metabolism. Therefore one of us (H. J. D.) went on a practically protein-free diet of about 1800 calories consisting of starch, sugar, centrifuged orange juice, lettuce, and definite amounts of salt solutions of Ca, Mg, Fe, Na, K, and Cl. This protein-free diet was continued for 54 days in which the carbohydrate at first was largely cooked corn-starch puddings and sugar; later, largely sugar, and finally cooked dried starch. A period of 8 days followed in which protein was taken in small amounts, and afterwards a second period of 9 days in which the protein-free diet was resumed. Urine specimens were collected in 24 hour samples. Determinations of the basal metabolism by the usual technique of Boothby and Sandiford were carried out daily.

The following determinations were carried out on the urine: volume, specific gravity, titratable acidity, total N, urea, ammonia, creatinine, creatine, uric acid, amino acid, total S, total sulfates, inorganic sulfates, neutral and ethereal S, inorganic and total phosphates, Folin sugar, fermentable sugar, and Mg, Ca, K, Na, and Cl. Blood samples were obtained at frequent intervals and determinations of blood sugar, non-protein N, urea N, creatinine N, amino acid N, and uric acid N, and Cl (the latter both on plasma and whole blood) showed these constituents normal both

<sup>2</sup> Boothby, W. M., Sandiford, I., Sandiford, K., and Slosse, J., *Ergebn. Physiol.*, 1925, xxiv, 728.

at the beginning of the experiment, later when the protein metabolism had fallen to a very low level, and after the administration of thyroxin.

On the protein-free diet all the urinary constituents studied remained constant except that there was a marked decrease in urea nitrogen with a parallel decrease in total N while the S and P tended to follow the N excretion. When the N metabolism was lowered approximately to a minimal level by the protein-free diet, thyroxin was administered which resulted in a temporary increase of the N excretion, the maximum being reached on the 9th day. At this point thyroxin was administered to keep the elevation of the basal metabolic rate constant and the N excretion fell almost to the original level. That thyroxin causes no toxic breakdown of protoplasm is further shown by the constancy of the creatinine excretion and the absence of any significant changes in any of the nitrogenous or inorganic constituents of the urine except that of urea. The essential points of Folin's theory of protein metabolism are confirmed. Likewise the earlier investigations of Boothby, Sandiford, Sandiford, and Slosse,<sup>2</sup> which show that a shift upward in the thyroxin concentration causes an increase in the breakdown of deposit protein which returns to normal when the organism is adjusted to the change, are confirmed.

**THE PERCENTAGE VARIATION OF THE NITROGEN PARTITION  
PRODUCTS IN THE URINE AS THE RESULT OF A PRO-  
LONGED PROTEIN-FREE DIET, TOGETHER WITH  
THE EFFECT THEREON OF THYROXIN AND  
SUBSEQUENT PROTEIN FEEDING.**

By IRENE SANDIFORD, KATHLEEN SANDIFORD, HARRY J.  
DEUEL, JR., AND WALTER M. BOOTHBY.

*(From the Section of Clinical Metabolism, Mayo Foundation, Rochester,  
Minnesota.)*

As pointed out by Folin the fluctuations of the total N are produced almost entirely by changes in the urea N fraction and it is the only nitrogenous substance which suffers a relative as well as an absolute diminution with a decrease in the total protein metabolism. In our experiment (see previous abstract) at the lowest level of N elimination, 1.75 gm., the urea N was only 43 per cent of the total.

The absolute values of the total creatinine, uric acid, and amino

acid N showed no significant fluctuations throughout the experiment but when expressed in percentages of the total N they are markedly increased as the total N decreases. The percentage of creatinine N rose to the height of 33 per cent of the total N on the day of lowest N elimination and was nearly equal to the percentage of urea N. The parallelism existing between the curves representing the percentages of creatinine, uric acid, and amino acid N is very striking and supports Folin's contention that these three substances indicate true endogenous protein metabolism. With the exception of the early part of the experiment when there is a decrease in the amount of ammonia eliminated as the total N decreases, the ammonia N remains constant in absolute amount and the percentage curve is similar to that for creatinine. Since the ammonia is concerned with maintaining the equilibrium between the excretion of acids and bases, this would indicate that no disturbance to this mechanism occurred as a result of the protein-free diet or the subsequent thyroxin administration. The rest N varied between 0.2 and 0.6 gm. and is indicative of the accuracy of the analytical data. The only significant fact that can be deduced from this is that thyroxin with an increased metabolism of stored protein does not cause the formation of any larger amount of any unknown nitrogenous waste product than occurs after the ingestion of ordinary protein food.

Calcium and magnesium show very slight fluctuations and none of any considerable magnitude; there are slight variations in the potassium which in general follow the more marked variations of sodium. The sum of all bases expressed in cc. of normal solution shows daily fluctuations of considerable magnitude which are caused in large part by changes in the elimination of sodium. However, the curve representing the sum of the acids (excluding the amino acids) almost exactly mirrors that of the bases.

#### **THE EFFECT OF EXERCISE ON BREATHING IN EXPERIMENTAL ALKALOSIS PRODUCED BY INGESTED SODIUM BICARBONATE.**

By ETHEL RONZONI.

*(From the Laboratories of Biological Chemistry and Internal Medicine,  
Washington University, St. Louis.)*

The average of three control experiments on three different individuals shows an accumulation of lactic acid during a period of

from 3 to 5 minutes strenuous exercise, stair climbing, sufficient to raise the blood lactic acid from 22 mg. to 125 mg. per 100 cc. of blood, producing a measured fall in pH of from 0.2 to 0.25 pH. The  $\text{CO}_2$  tension at this time, 2 to 3 minutes after exercise, is somewhat below the normal. During this early period after exercise when the lactic acid of the blood and the H ion concentration are still increasing, the respiration volume which has during exercise increased ten times drops off suddenly to seven times the normal respired volume.

If before equal amounts of exercise, enough sodium bicarbonate (30 gm.) is ingested to raise the pH from an average normal level of 7.38 (four cases) to 7.68 the  $\text{CO}_2$  tension is raised on an average of 9 mm. and the respiration is depressed about 10 per cent of its total volume.

The result of exercise is to cause an almost equal increase in rate and volume as that observed in the control experiments, in two cases the rate was slightly less and the volume greater, in one case the rate was increased more but the volume showed the same increase, and in the fourth case the increase in both volume and rate checked with the control experiments. In all cases the dyspneic condition of the subject as judged from the appearances and subjective symptoms was decidedly worse.

The change in pH due to an accumulation of a similar amount of lactic acid brought the pH back almost to its normal level of 7.38 but in no case below that level. The  $\text{CO}_2$  tension was below normal and the oxygen saturation above normal in all cases but one.

With an alkaline pH and no oxygen-want it is difficult to explain the initiation of respiratory stimulation unless we assume the adjustment of the respiratory center to an alkaline pH, or some reflex mechanism; once initiated it may be kept up by the mechanism suggested by Gesell, that is, the increased activity of the respiratory cells increases the  $\text{CO}_2$  tension or lactic acid within the center resulting in stimulation due to its inability to diffuse out. We also fall into difficulties when we try to explain the sudden drop in rate and volume at the end of exercise when the lactic acid and H ion concentration are still increasing and the  $\text{CO}_2$  tension is also increasing. Fatigue of the center is a possible explanation but this seems unlikely when we consider the short period of

exercise. The removal of some reflex stimulation seems to be the only possible explanation left.

### CARBON DIOXIDE TENSION OF CEREBROSPINAL FLUID.

By A. T. SHOHL AND S. KARELITZ.

(From the Department of Pediatrics, Yale University, School of Medicine, New Haven.)

By  $\text{CO}_2$  absorption curves, spinal fluid was found not to differ from a corresponding concentration of  $\text{NaHCO}_3$  in 0.9 per cent  $\text{NaCl}$ . The  $\text{pK}'_1$  value thus determined was less accurate than that calculated from the recently published activity coefficient of Hastings and Sendroy according to the equations

$$\begin{aligned} (1) \quad & \text{pK}'_1 = \text{pK}_1 - 0.5\sqrt{\phantom{x}} = 6.13 \text{ and} \\ (2) \quad & \text{p}_{\text{am}} = \text{pK}'_1 + \log R \end{aligned}$$

By the equation

$$(3) \quad \text{H}_2\text{CO}_3 = \text{C} \frac{1}{R + 1}$$

the value of  $\text{H}_2\text{CO}_3$  in spinal fluid is found to be higher than that of venous blood. The normal tension of  $\text{CO}_2$  in cerebrospinal fluid is approximately 5 to 10 mm. higher than that calculated for blood with values of about 45 to 50 mm.

### THE BIOCHEMICAL OXIDATION OF FATTY ACIDS.

By H. GREGG SMITH.

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.)

In 1909, Leathes expressed the theory that the body introduces ethylene linkages in the long chain fatty acids in order to form points of weakness at which these acids are first oxidized, forming two or more short chain acids which are subsequently oxidized in the ordinary manner, beta-oxidation. This idea has been generally accepted without question. The indirect evidence which is available does not support the theory, but is questionable and the present work is intended to obtain direct evidence for or

against the theory. If the short chain mono- and dibasic acids, formed in the laboratory oxidation of the unsaturated acids, are fed to animals and are utilized, the theory stands; if they are not burned, the theory lacks direct support and falls. Azelaic acid, the most common of the dibasic acids produced from unsaturated acids, and pelargonic acid, produced in the oxidation of ordinary oleic acid, were fed to dogs. Pelargonic acid was completely burned, but 40 per cent of the azelaic acid was excreted, giving partial evidence that the Leathes theory lacks direct support.

#### LIPID EXCRETION BY DOGS ON A LIPID-FREE DIET.

By WARREN M. SPERRY.

*(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, University of Rochester, Rochester, New York.)*

Previously reported work has indicated that the lipids found in the feces of dogs on a normal diet consist of a basal excretion, represented by that occurring with lipid-free diets, together with a smaller amount of diet lipid which has either escaped absorption or has been absorbed and reexcreted. Because of the value which a thorough knowledge of this basal excretion might have in helping solve problems of lipid metabolism, it seemed desirable to subject it to a rather detailed examination. About 60 analyses of the feces of animals receiving no lipid in their food have failed to show a single case in which fatty substances were absent or nearly absent and, moreover, the composition of the material excreted by different animals has been found to be quite uniform throughout the work. In order to eliminate any possibility of error due to poor demarcation, experiments have been carried out over long periods of time and even after 5 weeks lipids continued to be excreted in nearly as large amount and of the same composition as at the beginning. Analysis has shown that the fatty acids, which make up about 60 per cent of the basal lipid excretion, are composed in the main of palmitic, stearic, and very probably oleic acids and it is, therefore, difficult to explain this as a real excretion of waste products; and for the same reason it seems hardly probable that these fecal lipids can reach the intestine by way of the bile, since, if they did, we would expect them to be reabsorbed. This view is supported by preliminary experiments

with bile fistula dogs receiving strictly lipid-free diets. Lipids continued to be excreted by such animals in larger amounts than the same animals excreted while they were still normal.

#### SOME EFFECTS OF POSTERIOR PITUITARY ABLATION IN THE RAT.

BY G. L. FOSTER AND PHILIP E. SMITH.

*(From the Departments of Biochemistry and Anatomy, University of California, Berkeley.)*

An operation has been developed by one of us which makes it possible to ablate either the entire hypophysis, or only its posterior lobe (neural and intermediate lobe) component, leaving the capsule of the gland, the pituitary stalk, and the pars tuberalis intact. Any brain injury appears to be precluded. In the posterior lobe ablation the anterior pituitary is also usually partly removed, a sufficient amount being left, however, so that no anterior lobe deficiency is displayed by the animal. The operative approach leads directly to the nasal surface of the sphenoid between the medial pterygoid laminae without opening the oral cavity.

In six animals the posterior lobe has been either completely or nearly completely ablated as shown by a study of serial sections of this region. Growth, the sex cycles, and the capacity to bear young appeared to be not impaired; nor, as shown by the autopsy, were there significant weight changes or structural alterations in the thyroids, adrenals, or ovaries.

Studies were made on these animals of the basal metabolism and the specific dynamic action of glycocoll injected intraperitoneally, using a closed circuit respiration apparatus adapted for short periods. The basal metabolism in calories per kilo per hour was found to be not different from that of the normal animal, but the specific dynamic action of glycocoll, while well marked in the normal rat, was entirely absent in the case of the operated animals. Daily injections of posterior lobe extract for several days brought about the normal response to amino acid injection in the animals which had undergone posterior lobe ablation. Cessation of the injections for several days resulted in the disappearance of the amino acid stimulation, which was again restored by a renewal of the posterior lobe injections.



**THE EFFECT OF COMPENSATORY HYPERTROPHY ON THE FAT,  
CHOLESTEROL, AND PHOSPHATIDE CONTENT OF THE  
SUPRARENAL GLAND.**

By EMIL J. BAUMANN.

(*From the Chemistry Laboratory, Montefiore Hospital, New York.*)

The suprarenal glands of rabbits were removed under ether anesthesia in two stages. The interval between removal of right and left glands varied between 7 and 73 days. The glands were analyzed for their fat (total ether-alcohol extract soluble in chloroform minus cholesterol and phosphatides), cholesterol, and phosphatide contents.

In normal animals the left suprarenal gland is almost always larger than the right (about 10 to 20 per cent). The per cents of cholesterol and phosphatides are greater in the smaller (right) glands so that the total amount of cholesterol and phosphatides is about the same in both glands.

Compensatory hypertrophy of the left suprarenal usually results in an increase in the per cent of phosphatides and consequently in the total amount also. After complete compensation the per cent of phosphatides seems to decrease although the total amount is somewhat higher.

Considerable variation in the cholesterol content of suprarenal glands of different animals was noted. The differences in the composition of right and left suprarenals of rabbits in the hypertrophy experiments were of the same magnitude as the variations in normal right and left suprarenals. If cholesterol were a *specific* element in suprarenal gland function, as has been maintained so often, we should then expect an unequivocal decrease in the per cent of cholesterol during the active stage of hypertrophy of the gland. This we were unable to show.

**THE INTERMEDIARY METABOLISM OF THE AROMATIC AMINO  
ACIDS.  
PHENYLALANINE.**

By NOEL F. SHAMBAUGH\* AND HOWARD B. LEWIS.

(*From the Laboratory of Physiological Chemistry, University of Michigan, Ann Arbor.*)

Phenylalanine has been administered *per os* and subcutaneously

\* Medical Fellow of the National Research Council in Physiological Chemistry.

to rabbits in doses of approximately 1.0 gm. per kilo and the changes in the blood and urine have been studied. 3 hours after the administration of the phenylalanine, the phenol content of the blood as determined by Benedict's *p*-nitroaniline method had increased to about twice the normal value. The total phenols of the urine (Folin-Denis) increased indicating that about 10 per cent of the aromatic nucleus of the phenylalanine had been eliminated in the urine as phenols. There was no corresponding increase in urinary amino acid nitrogen. The formation of the phenols was not due to intestinal microorganisms since the excretion of phenols was as great after subcutaneous injection of phenylalanine as after oral administration. The increased phenol content was due in part to the presence of an *o*-diphenol derivative which gave the typical reaction of *o*-diphenols with ferric chloride. The substance which gave the reaction has been extracted from the urine and did not contain amino acid nitrogen. After the administration of tyrosine, the phenol content of blood and urine increased, but no evidence of the presence of an *o*-diphenol was obtained. The results are considered to indicate that in the oxidation of the aromatic group of phenylalanine, a diphenol is formed as an intermediary product, and that tyrosine and phenylalanine do not follow the same paths in intermediary metabolism in the organism of the rabbit.

**THE EFFECT OF ORANGE JUICE ON THE CALCIUM, PHOSPHORUS,  
MAGNESIUM, AND NITROGEN RETENTION AND URINARY  
ORGANIC ACIDS OF GROWING CHILDREN.**

BY MARGARET S. CHANEY AND KATHARINE BLUNT.

*(From the Department of Home Economics, University of Chicago, Chicago.)*

A study was conducted on two growing girls, 10 and 11 years old, to determine the effect of orange juice on calcium, phosphorus, magnesium, and nitrogen metabolism.

The basal diet consisting of oatmeal, rice, flour, bread, potato, sugar, oleomargarine fat, lean beef, and whole milk powder was ingested for a preliminary adjustment period and a 3 day collection period. Then orange juice, 600 to 700 cc. daily, was added to the diet; another adjustment period and collection period followed. After an interval of 2½ months the experiment was again performed on the same girls.

Calcium assimilation was decidedly benefited when oranges formed a part of the diet. The increased retention was considerably greater than the calcium added in the oranges and greater than might be expected from a stimulus to retention caused by a larger calcium intake.

The increase in phosphorus retention was even more marked than that of calcium, more than three times as much phosphorus both in gm. and percentage of intake, being assimilated by the body when orange juice was added to the diet.

The magnesium retention was also increased, although to a less marked extent than that of calcium and phosphorus.

Nitrogen assimilation was greater when orange juice was ingested, even though the nitrogen intake was not altered.

Urinary ammonia was decreased, and urinary pH and organic acids increased, thus confirming the work of Blatherwick and Long. The increase of organic acids amounted to approximately 7 per cent of the citric acid of the orange juice ingested.

A marked increase in the children's weight was observed.

#### CALCIUM, PHOSPHORUS, AND MAGNESIUM METABOLISM IN THE RAT.

By GRACE MEDES.

*(From the Department of Physiological Chemistry, University of Minnesota Medical School, Minneapolis.)*

Pedigreed rats 32 to 48 days old were fed for varying periods on weighed portions of diets of casein, starch, sugar, vitamins A (extracted from spinach) and B (Harris yeast vitamin) and salt mixtures in which the ratios of P to Ca, P to Mg, and Ca to Mg were varied in the different experiments.

At the close of each experiment the rats were ashed and the Ca, Mg, and P determined. The excreta and samples of the food were also ashed and the same elements determined and from these data the composition of rats at the beginning of the experiments was estimated.

Lantern slide tables were presented giving the metabolic balance in Ca, Mg, and P showing the effect of high and low dietary Ca, Mg, and P on the metabolic balance of these elements.

**THE PHOSPHORUS CONTENT OF THE BODY IN RELATION TO AGE, GROWTH, AND FOOD.**

BY H. C. SHERMAN AND E. J. QUINN.

*(From the Department of Chemistry, Columbia University, New York.)*

The phosphorus content of the body of the normal white rat is about 0.34 per cent at birth and increases during growth and development, at first rapidly and then more gradually, to from 0.60 to 0.78 per cent in the full grown animal. Thus the body during growth multiplies its birth phosphorus about twice as many fold as its birth weight. The gain of phosphorus by the body has been studied in detail with reference to its relations to the age and general growth, the calcification of the bones as indicated by increase of body calcium, the breeding records of the females, and the influence both of dietary deficiencies and of changes in the character of the food within normal limits.

**STUDIES ON INSULIN.**

BY N. R. BLATHERWICK, L. C. MAXWELL, JOHN BERGER, AND MELVILLE SAHYUN.

*(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa Barbara Cottage Hospital, Santa Barbara.)*

*A Simpler Method of Preparing Purer Insulin.*—In this method, the procedure of Scott and Best<sup>3</sup> is followed up to the point where they precipitate with  $(\text{NH}_4)_2\text{SO}_4$ . In place of the ammonium salt we saturate by adding 22 gm. of NaCl per 100 cc. The salt precipitate is dissolved in the original volume of water and again saturated with NaCl without changing the reaction. This moist precipitate is dissolved in sufficient 95 per cent alcohol to make a concentration of 75 per cent, filtered or centrifuged to remove insoluble matter, and the insulin is again precipitated by adding to two volumes of ether. The ether precipitate is dissolved in water and brought to the isoelectric point with NaOH. After the third isoelectric precipitation from water, a yield of about 1000 clinical units per kilo of pancreas is obtained. The nitrogen amounts to approximately 0.005 mg. per unit. This insulin precipitates at a pH of about 5. The second saturation with NaCl

<sup>3</sup> Scott, D. A., and Best, C. H., *Ind. and Eng. Chem.*, 1925, xvii, 238.

appears to remove most of the contaminating pH 7 protein. Further saltings do not effect a greater purification.

*Purification of Insulin by Fractional Precipitation with Alcohol.*—A less pure insulin is obtained when but one salt precipitation is made in the above method. This crude insulin, when dissolved in alcohol, precipitated with ether, and precipitated once at the isoelectric point from water, contains at least two substances. These can be rather completely separated utilizing their different solubilities in alcohol. If the isoelectric precipitate is dissolved in a minimum amount of water and NaOH, alcohol added to a concentration of about 75 per cent (hydrometer), and HCl added to a pH of about 6.6, a dirty, meaty-looking precipitate is formed. This material is relatively impure. It carries most of the red-brown color of the original solution. Upon the addition of absolute alcohol to the supernatant fluid from this precipitate in amount to make a concentration of about 92 per cent alcohol, and upon adjusting to a slightly more acid reaction, a flocculent, white precipitate forms. This material is much purer than the other fraction and carries most of the activity. The results of a typical experiment were: mg. N per unit in the first fraction 0.050, in the second 0.0051. These observations confirm and extend the findings of Somogyi, Doisy, and Shaffer.<sup>4</sup>

*Dialysis.*—The material used in these experiments was extracted according to Collip's method. After concentration, and removal of fat with ether, the aqueous solution was saturated with NaCl. This precipitate was dissolved in alcohol and precipitated by adding to ether. The aqueous solution of this precipitate was dialyzed. Before dialysis the solutions were brought just through the isoelectric point with NaOH. Rather thin collodion membranes were used. Dialysis was continued for about 5 hours with three changes of distilled water. The first experiment showed that 4 per cent of the active material dialyzed. 63 per cent of the potency was found in the isoelectric precipitate of the residue and 33 per cent remained in the filtrate. The biuret reaction of the concentrated dialysate was a distinct pink in contrast with the violet color of the isoelectric material. Under the experimental conditions a precipitate

<sup>4</sup> Somogyi, M., Doisy, E. A., and Shaffer, P. A., *J. Biol. Chem.*, 1924, **1x**, 31.

formed within the sac during dialysis. This precipitate was removed and kept separate from the isoelectric material obtained from the residual fluid. The sac precipitate was less pure and somewhat more difficult to purify than the one obtained by isoelectric precipitation. Repeated precipitations at the isoelectric point brought the N content of both fractions to less than 0.005 mg. per clinical unit. The isoelectric point of these preparations when purified was about pH 6 (colorimetric). Dialysis permits a sharper precipitation at the isoelectric point due to the low salt concentration. Loss of potency is therefore not so great.

*Heating.*—Fairly crude or purified insulin in aqueous solution is precipitable by heat. The necessary conditions are: (1) the pH should be 4.0 to 4.2, (2) the solution should contain from 50 to 100 units per cc., and (3) NaCl content should be nearly 1 per cent. We heat at a temperature of 80°C. for about 1.5 hours. The precipitate which forms is usually colloidal and is not to be confused with an isoelectric precipitate. If conditions are right, the potency is practically regained in the heat precipitate and impurities remain in the filtrate.

## THE RELATION OF THE PARATHYROID GLANDS TO THE CALCIUM OF THE BLOOD.

By ISIDOR GREENWALD.

(From the Harriman Research Laboratory, The Roosevelt Hospital, New York.)

Although, by the addition of sodium citrate, it is possible to prepare simple saline solutions that will keep calcium phosphate in solution in quantities comparable to those found in serum, the solubility of calcium phosphate in serum is not due to the presence of citrate. Serum does not contain the requisite amount of citrate. Solutions of serum proteins, or of crystallized egg albumin, to which calcium chloride and sodium phosphate have been added furnish solutions containing calcium phosphate in quantities greater than those normally present in serum. However, it has also been found possible to prepare concentrated dialysates from serum that are protein-free yet contain, at pH ranging from 7.2 to 8.5, quantities of calcium phosphate far in excess of those pos-

sible in simple saline solutions. When such solutions are acidified, boiled for 1 hour, cooled, and adjusted to original volume and reaction, most of the calcium is precipitated and the filtrates contain only small amounts. It is suggested that it may be through the production of increased amounts of this diffusible, thermolabile substance that the parathyroid hormone operates to increase the calcium concentration of the serum and that it is because of the absence of this substance that the concentration of calcium in the serum of parathyroidectomized dogs decreases.

### THE PARATHYROID HORMONE.

By AXEL M. HJORT, H. B. NORTH, AND F. H. TENDICK.

*(From the Department of Chemical Research, Parke, Davis and Company, Detroit.)*

A study was made of the uniformity and constancy in action of extracts prepared identically from one lot of acetone-desiccated external bovine parathyroid glands. Ten normal dogs, both male and female (10 to 12 kilos), were employed. The experiments were performed at 2 week intervals to avoid possible complication of induced anemia. Two females passed through normal gestation periods during the course of the experiments. The effect of calcium-rich diets upon the response of the animals to the extracts was also studied. In every case save one, the extracts were given subcutaneously, the excepted case intravenously. One experiment was performed with each of two other groups of normal dogs to study the diurnal variations of the serum calcium, and the relationship between dosage and body weight.

Barring extremely young and old dogs no direct relationship between induced hypercalcemia and age, sex, weight, pregnancy, and diet was observed. Different dogs varied as much in their response to the same dosage of extract as the same dog at different times. In some cases, a very slight early transient rise in serum calcium followed intravenous administration of the extract; in others, no increase was induced.

The diurnal variation of serum calcium in fasting normal dogs may be as much as 10 per cent.

**THE CONTROL OF THE MUSCULAR RIGIDITY OF CATATONIC PRÆCOX PATIENTS BY PARATHYROID HORMONE.**

By JOSEPH M. LOONEY.

*(From the Biochemical Laboratory of the Sheppard and Enoch Pratt Hospital, Towson, Maryland.)*

In previous communications<sup>5,6</sup> from this laboratory it was shown that the creatine content of the blood varied with the muscular tension of the individual. In catatonic præcox patients showing marked rigidity there is an increase in creatine measured in per cent of non-protein nitrogen from 8.4 to 12.9. Various investigators have pointed out that there is an increase in guanidine excretion in cases of tetany and that there is also a decrease in the calcium content of the blood. Because of the close chemical relation between methyl guanidine and creatine it was felt that there might also be some change in the calcium content of the blood of these markedly catatonic cases.

In a series of eleven cases showing various degrees of rigidity a slight decrease in the calcium content of the blood was obtained. The average content was 9.1 mg. per 100 cc. The calcium content of the serum was increased from 0.4 mg. to 14.0 mg. by injections of parathyroid hormone (Collip). This figure was maintained for several days and there was a decrease in the muscular rigidity so that, whereas before the injections it was impossible to open the patient's hands, afterwards almost complete extension was obtained.

25 units of parathyroid hormone can be injected every 8 hours over a period of 5 days without danger.

I wish to express my appreciation to Dr. Collip and to the Eli Lilly Company for their courtesy in supplying me with the parathyroid hormone.

<sup>5</sup> Looney, J. M., *Am. J. Physiol.*, 1924, lxi, 639.

<sup>6</sup> Looney, J. M., *Am. J. Psychol.*, 1924, lv, 29.



**PLASMA PROTEINS IN EXPERIMENTAL ANHYDREMIA.**

By MEYER BODANSKY.

*(From the Laboratory of Biological Chemistry, University of Texas School of Medicine, Galveston, and the Department of Chemistry, Stanford University, California.)*

A study has been made of the plasma proteins in a variety of conditions associated with concentration of the blood. Following the administration of symmetrical diisopropyl hydrazine hydrochloride, the per cent decrease of the plasma volume may exceed 30, and is associated with an increased concentration of the plasma proteins which does not quite parallel the plasma volume decrease. Thus, in two dogs, decreases in plasma volume of 30 and 34 per cent were observed, whereas the protein concentrations increased but 25 and 31 per cent respectively, showing that a small amount of protein probably escaped from the blood to the tissues.

Similar but less marked changes were likewise observed in intoxications with hydrazine and 2-2'-azobispropane.

In pilocarpine anhydremia, concentration of the blood and of the plasma proteins may run parallel. In one case, however, in which hemorrhagic extravasation in the liver and kidneys was especially severe, the protein increase was only 4.0 per cent as compared with a 13.0 per cent decrease, from the normal, of the plasma.

On the other hand, in histamine shock, both the plasma volume and protein content diminish. Thus with plasma volume decreases of 38.0 and 14.0 per cent, protein decreases of 8.6 and 16.0 per cent respectively were observed, showing that in histamine shock the permeability of the capillary wall for proteins is much greater than in the other forms of anhydremia studied.

The albumin-globulin ratio of the blood decreased in all the forms of anhydremia studied with the exception of two cases of histamine shock where no significant variations from the normal were observed. An attempt was made to determine variations from the normal of the various globulin and albumin fractions by Howe's methods. No constant changes were observed in the anhydremic state except for fairly consistent increases of the fibrinogen and euglobulin fractions.

Except in hydrazine poisoning, concentration of the blood was always accompanied by a decrease in the carbon dioxide tension.

**THE USE OF THE HOPKINS-COLE GLYOXYLIC ACID REAGENT  
FOR THE QUANTITATIVE DETERMINATION OF FREE  
TRYPTOPHANE IN BLOOD.**

By C. A. CARY.

*(From the Research Laboratories of the Bureau of Dairying, United States  
Department of Agriculture, Beltsville, Maryland.)*

To determine the free tryptophane in the blood or the blood plasma of cows, the protein was removed as in the amino N procedure used in this laboratory<sup>7,8</sup> except that the trichloroacetic acid was omitted. The concentrations were done *in vacuo*. The aliquots, after the second kaolin treatment, were brought to about twice the volumes of the blood or plasma represented in them and precipitated with  $\text{H}_2\text{SO}_4$  (7 per cent by volume) and  $\text{HgSO}_4$  (10 per cent) by allowing to stand in ice box overnight. These precipitates were then filtered onto asbestos, washed with 5 per cent  $\text{H}_2\text{SO}_4$  and returned to the original vessels along with the asbestos mats. 20 cc. of freshly prepared glyoxylic acid reagent<sup>9</sup> and 4 drops of a solution of 25 per cent mercuric sulfate in 10 per cent  $\text{H}_2\text{SO}_4$  were added to each. After 48 hours at room temperature these mixtures were filtered and compared with tryptophane standards similarly precipitated and treated. The color, recovery of added tryptophane, and agreement in duplicate determinations were very satisfactory. The use of  $\text{CCl}_3\text{COOH}$  or other protein precipitants in addition to the above procedure did not alter the results. A portion of the color (15 to 20 per cent) may be due to indole derivatives other than tryptophane in the blood or to decomposition products of tryptophane formed in the procedure. No correction for this is made in the data given in the paper following.

<sup>7</sup> Cary, C. A., *J. Biol. Chem.*, 1920, xliii, 477.

<sup>8</sup> Cary, C. A., and Meigs, E. B., *J. Agric. Research*, 1924, xxix, 603.

<sup>9</sup> Reagent contains 20 cc. of  $\text{H}_2\text{SO}_4$  solution (21 cc. of 95 per cent  $\text{H}_2\text{SO}_4$  + 5 cc.  $\text{H}_2\text{O}$ ) and 1 cc. of glyoxylic acid solution (100 cc. of saturated oxalic acid and 6 gm. of 5 per cent sodium amalgam).

**THE FREE TRYPTOPHANE IN COW'S BLOOD AND ITS UTILIZATION BY THE MAMMARY GLAND.**

By C. A. CARY.

*(From the Research Laboratories of the Bureau of Dairying, United States Department of Agriculture, Beltsville, Maryland.)*

In eleven samples of cow's blood the free tryptophane varied from 0.95 to 1.47 mg. per 100 cc.; in seven samples of plasma it varied from 0.71 to 1.35 mg. This tryptophane is taken out of the plasma of the blood and used by the mammary gland in the secretion of milk. This was shown by comparing the concentrations of it in plasma from samples of blood taken simultaneously from the jugular and mammary (abdominal subcutaneous) veins. In two experiments with milking cows the free tryptophane in the mammary blood plasma was 19 per cent and 17.7 per cent lower than in the jugular. With three cows that were not milking these differences were 0.7, 3.1, and 8.1 per cent, with a flow of blood through the glands that was only a fraction of that in the milking cows.

**THE NORMAL URINARY PIGMENT.**

By DAVID L. DRABKIN.\*

*(From the Laboratory of Physiological Chemistry, Yale University, New Haven.)*

The normal pigment of urine, commonly termed *urochrome*, was studied under conditions in which the presence of extraneous pigments was eliminated from the diet and such substances as urobilin and hematoporphyrin were excluded. Most of the experiments were performed upon dogs, but the urines of rats and humans were also studied. The daily output of pigment was determined colorimetrically. The standard used was a mixture of dyestuffs which simulated closely the color and fluorescence of urine. The determinations were expressed empirically in units.

It was found that, under controlled conditions, the quantity of urinary pigment eliminated from day to day is constant. The output upon a practically colorless food mixture, used as the standard diet, was determined. A study of the effect of changes in

\* Medical Fellow of the National Research Council.

the character of the diet upon the color of the urine indicated that the amount of urinary pigment is essentially independent of the diet. The administration of alkalies decreased the quantity of urine pigment. Marked increases in pigment output followed the administration of acids, fasting, and, above all, the augmented metabolism produced by thyroxin, adrenalin, and phlorhizin. In a severe case of Graves' disease the output of the urinary pigment from day to day was found to be a good index of the patient's metabolic rate and clinical progress. The volume of urine was found to vary, in a general way, with the amount of pigment, but diuresis *per se* caused no change in pigment output.

That the normal urinary pigment is an endogenous product is further suggested by the comparative study of several species. Calculated upon a kilogram basis, a rat eliminates in the urine 7.9 times the amount of pigment of an adult man, 3.6 times that of a medium sized dog, 2.7 times that of an infant, and 1.4 times that of a 2 months old puppy. Furthermore, the figures for the pigment output of the different species plotted against the surface area give a straight line.

#### FRACTIONAL PRECIPITATION OF PROTEIN IN URINE.

By ANTON R. ROSE AND W. G. EXTRON.

(From the Laboratory of The Prudential Insurance Company of America, Newark.)

A procedure for fractional precipitation of protein in urine by ammonium sulfate has been devised, and applied to random proteinuria specimens, also to some controlled cases. The precipitates were measured by the skopometer, a turbidity meter with "disappearance criteria." Globulin occurs in most specimens of proteinuria ranging from 0 to 90 per cent of the total protein. In no specimen containing 100 mg. or more of protein per 100 cc. did the globulin constitute one-third of the total protein and globulin-free specimens occurred in both protein-rich and protein-poor samples. In some cases the globulin-albumin ratio was constant throughout the 24 hour day; in others it varied considerably.

**HYPOGLYCEMIA WITH CONVULSIONS IN PHLORHIZIN DIABETES.**

By M. WIERZUCHOWSKI.

*(From the Department of Physiology, Cornell University Medical College, New York City.)*

Ten dogs were fasted and were daily phlorhizinized until they became comatose. In three such animals convulsions began during the comatose state when the blood sugar level reached 0.06 per cent. Oral administration of glucose relieved the convulsions in 20 minutes. At the same time the ketone bodies disappeared from the breath and urine and also from the blood, as indicated by the rise in its carbon dioxide-combining power; coma disappeared completely, urinary nitrogen fell (Deuel and Chambers), and the dog reacted as does one in normal fasting. These beneficial phenomena were due to an increase in the blood sugar level, for as the blood sugar again decreased, ketone bodies reappeared in the breath and urine, the carbon dioxide-combining power of the blood fell, coma again intervened, and finally, when the blood sugar fell to a level somewhat below that observed in the first instance, convulsions took place as before. About 80 per cent of the glucose ingested was recovered as extra sugar in the urine. The two lethal syndromes, hypoglycemic convulsions and coma, were therefore completely relieved by glucose administration to phlorhizinized dogs. Whether the ingested glucose was oxidized or not will be shown in a subsequent report.

**A CHEMICAL AND ELECTRONIC BASIS FOR THE THEORY OF ANTIKETOGENESIS.**

By EDWARD S. WEST.

*(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis.)*

According to the modern theory of antiketogenesis in the animal body, acetoacetic acid or other ketogenic molecule is condensed with a sugar or sugar derivative to form a compound which has a higher reduction potential, or is more easily oxidized than the unchanged ketogenic molecule.

It has been found that methylene-, ethylidene-, benzylidene-, vanillylidene-, and furfurylidene-bis-acetoacetic ester (acid), and

also ethyl  $\alpha'$ -acetoacetic ester- $\alpha$ -methyl- $\beta$ -furan carboxylate and a condensation product of glucose and acetoacetic ester are much more powerful reducing agents than unchanged acetoacetic ester (acid). In all of these cases the carbon atom of the acetoacetic acid chain which is linked to two mobile hydrogen atoms has been linked to carbon of the condensing molecule by one valence and still retains one mobile hydrogen atom. Condensation products of acetoacetic ester (acid) in which this carbon atom is linked to carbon of the condensing molecule by two valences and retains no mobile hydrogen show much less reducing power.

From a consideration of the electronic configuration of acetoacetic acid and its condensation products the latter should possess, as actually found, the higher reduction potentials.

The theory of antiketogenesis as put forward by Woodyatt, Shaffer, etc., therefore, has a firm basis of fact from the standpoint of the ease of oxidation of condensation products of acetoacetic ester (acid).

#### THE EFFECT OF KETOSIS ON INSULIN ACTION IN THE RABBIT.

By ELMER L. SEVRINGHAUS.

*(From the Department of Physiological Chemistry, University of Wisconsin, Madison.)*

The occurrence of ketosis due to carbohydrate deprivation in the healthy adult human produces a reduced rate of glucose utilization following a simple meal. This result is apparent following the first intake of carbohydrate, after which it disappears. To determine the nature of the responsible factors rabbits were injected with insulin and simultaneously with acetone or with sodium acetoacetate. Controls were injected with either the insulin or the ketone body. The rabbits were fasted for 24 hours before use. Controls and test animals were of nearly the same weight. The insulin dosage was 3 clinical units per 2 kilos. Blood sugar concentration was determined in venous blood from the ears by the Folin-Wu method or by Gibson's adaptation for 0.1 cc. samples. Acetone was prepared by redistillation. Sodium acetoacetate was prepared from the ethyl acetoacetate by saponification with alkali, preparation of the soluble barium salt, and decom-

position with sodium sulfate to remove barium. The product was not toxic except in the effect described below.

The animals injected with acetone showed no more variation in blood sugar level than untreated animals. Insulin produced hypoglycemia in a typical manner. Acetone dosage was from 0.05 to 0.4 cc. per kilo. With the higher doses general depression of the animal was seen. The animals injected with sodium acetoacetate showed a tendency to hyperglycemia. When insulin was injected simultaneously there was a retardation in the decline of the blood sugar level and the degree of hypoglycemia was less marked than in control animals, with insulin alone. The effects of sodium acetoacetate on insulin action are not permanent. The effect of acetoacetate on insulin *in vitro*, and the results of injection of sodium beta-oxybutyrate are being sought. These results are believed to be important in disclosing the significance of insulin therapy in non-diabetic ketosis and the relative inefficiency of insulin in diabetic coma.

#### THE EFFECT OF GLUCOSE AND INSULIN INJECTION UPON ARTIFICIAL KETOSIS OF NORMAL AND DIABETIC DOGS.

By THEODORE E. FRIEDEMANN, M. SOMOGYI, AND P. K. WEBB.

(From the Department of Biological Chemistry, Washington University  
School of Medicine, St. Louis.)

Freshly prepared solutions of sodium acetoacetate were injected intravenously into dogs at approximately constant rates for periods of 6 to 20 hours. Up to a rate of injection of about 2 mm per kilo per hour hardly perceptible amounts of acetoacetate were excreted.<sup>10</sup> Up to a rate of 4 or 5 mm about 90 per cent was burned, only 5 to 9 per cent being excreted in the urine, and less than 1 per cent exhaled as acetone. Injections of insulin and glucose did not increase the tolerance.<sup>11</sup>

Two depancreatized dogs showed a normal acetoacetate tolerance, in spite of typical D:N ratios. The injections of phlorhizin into two normal dogs during the course of the experiment failed to affect their acetoacetate tolerance. The tolerance was definitely lowered after prolonged fasting and phlorhizin treatment,

<sup>10</sup> Wilder, R. M., *J. Biol. Chem.*, 1917, xxxi, 59.

<sup>11</sup> Burn, J. H., *J. Physiol.*, 1925, lx, p. xvi.

being in one case thus reduced to 50 per cent and in another to 70 per cent of the normal. Injection of insulin and glucose into one of these animals resulted in normal tolerance after 3 hours.

Base was excreted as  $\text{NaHCO}_3$  coincident with and paralleling the acetoacetate injection. The base excreted was almost equal to the amount of acetoacetate disposed of by the body. Analyses of the blood showed a high alkalemia, but only a slight increase of acetone bodies. The tissues contained only small quantities of acetone bodies. The fact that the base was excreted and that only small amounts of the acetone bodies were present in the tissues at autopsy indicates that the acetoacetate was burned.

With the possible exception of the fasting phlorhizinized dogs, the results appear to indicate that the dog is able to oxidize acetoacetate without intervention of carbohydrate metabolism, and that this animal is consequently not suited to the study of the relation of carbohydrate to antiketogenesis, which relation is believed to exist in man.

#### THE EFFECT OF ACIDOSIS ON THE SUGAR METABOLISM.

By ALFRED E. KOEHLER.

*(From the Department of Medicine, Henry Ford Hospital, Detroit.)*

It has been frequently observed that those physiological upsets associated with an acidosis such as anoxemia or anesthesia usually cause an increase in the fasting blood sugar.

We have noted that the production of a simple acidosis by the ingestion or intravenous injection of acids or acid-producing substances such as  $\text{H}_3\text{PO}_4$ ,  $\text{NH}_4\text{Cl}$ , or  $\text{CaCl}_2$  resulted in a definite increase in the fasting blood sugar of normal human subjects. A change in the venous blood reaction from approximately pH 7.40 to 7.20 was associated with an increase in the fasting blood sugar of about 30 to 40 mg. per 100 cc.

A change by the same means of similar magnitude in the blood pH of several diabetic patients studied under the same dietetic conditions resulted in a much greater and more variable increase in the fasting blood sugar. It was also noted that the blood pH of some diabetic patients was very much more easily shifted in the acid direction by even small amounts of acid intake.



In the several diabetic patients with acidosis (blood pH 6.80 to 7.20) huge doses of insulin (100 to 300 units in 12 hours) had no marked effect, but when alkali in the form of 3 per cent  $\text{NaHCO}_3$  solution was administered intravenously in just sufficient quantities to return the blood reaction to normal, the blood sugar decreased very rapidly.

The depressing effect of acidosis upon the sugar metabolism may be explained at least in part by a variation of Murlin's experiments on the inactivation of insulin by glucose. We have noted that insulin (Lilly) incubated at body temperature with a 0.25 per cent glucose solution was only mildly inactivated at a pH of 7.40 and markedly at 6.80. It is thus possible that the acidotic depression of the sugar metabolism is related to an acceleration of the inactivation of insulin by glucose due to increased H ion concentration, especially when there is an overproduction or mobilization of the latter.

#### STUDIES ON CAPILLARY BLOOD SUGAR.

By H. V. GIBSON.

*(From the Psychiatric Institute, University of Wisconsin, Madison.)*

Since previous investigations of blood sugar in the human have been made chiefly on venous blood, more information concerning arterial sugar phenomena is pertinent. Foster has shown that capillary blood approximates arterial blood in its sugar content. A suitable micro method for blood sugar was devised, using 0.1 cc. of blood and a modification of the Folin-Wu colorimetric method. The maximum error of the method is 3 per cent.

Studies were made on capillary blood following oral administration of glucose and the data plotted. Females during the child-bearing period were found to have different shaped sugar curves from those of males, and of females past menopause. Pregnant females were found to have this variation exaggerated. A peculiar type of diabetes in pregnancy was found to have a normal sugar curve while showing acetone and glucose in the urine. This condition disappeared following delivery. A fairly constant notching occurred in the ascending limb of the curve, probably due to beginning storage in the liver. Venous sugar was found

to exceed arterial sugar in the first part of the curve of a diabetic. Hypopituitarism was associated with a low arterial concentration, demonstrating that in this type of disorder, the marked storage occurs before the sugar reaches the peripheral circulation. Acromegalic patients showed abnormally high sugar curves in the capillary blood.

#### A QUANTITATIVE ANALYSIS OF THE FATE OF SUGAR IN THE ANIMAL BODY.

BY CARL F. CORI AND GERTY T. CORI.

*(From the State Institute for the Study of Malignant Disease, Buffalo.)*

An attempt has been made to follow the fate of sugar in the animal body on a quantitative basis. The experiments were carried out as follows. Male rats of 120 to 150 gm. body weight were fasted for 48 hours. The urine of the last 24 hours was collected quantitatively for N determinations. The fasting metabolism of the rats was then determined for a period of 2 to 3 hours in a metabolism apparatus of the Haldane type. The temperature of the animal chamber varied between 27 and 28°C. The rats were then fed a known amount of glucose solution by stomach tube and immediately replaced in the metabolism apparatus. After 4 hours the rats were killed and the intestinal tract quickly removed. The carcass was frozen with compressed CO<sub>2</sub>, minced, and introduced into boiling 60 per cent KOH for glycogen determinations. The amount of glucose remaining in the intestine was determined and subtracted from the amount of glucose fed. The difference between these two values was the amount of glucose absorbed during the metabolism period. The amount of sugar oxidized was calculated from the non-protein R.Q. From the amount of glycogen found, the amount of glycogen present in rats fasted for 48 hours had to be subtracted. Nine rats, fasted for 48 hours, contained as an average 0.116 per cent glycogen, maximum 0.132, minimum 0.098 per cent.

The amount of glucose oxidized plus the amount of glucose converted into glycogen amounted to 89.7 per cent of the total amount of glucose absorbed (average of ten experiments). The ratio, glucose oxidized:glycogen formed was of the order 1:1.3.

In a second series of experiments insulin was injected simultaneously with the sugar feeding. 90.9 per cent of the absorbed sugar was accounted for (average of nine experiments). The ratio, glucose oxidized:glycogen formed was of the order 1:0.9.

These experiments disprove the current theory that sugar is converted into an unknown substance during insulin action. Definite evidence was obtained that insulin increases the sugar oxidation.

### THE METABOLISM OF GALACTOSE.

### III. THE INFLUENCE ON THE TOLERANCE OF DISTURBED ENDOCRINE FUNCTION.

By ALLAN WINTER ROWE.

(From the Department of Chemistry, Evans Memorial, Boston.)

Galactose is a sugar not foreign to the human economy and yet of so low a tolerance that test meals are never productive of gastrointestinal disorders. The sugar is given to the fasting patient and urine collected at 2 hour intervals, both before (control) and after the test meal. Tolerance level is defined as the smallest amount of galactose which will produce a brief appearance of sugar in detectable amount in the urine. The higher normal tolerance of the female (40 gm.) over that of the male (30 gm.) is intrinsic.

The present report deals with the application of this test to nearly 400 subjects, most of whom had a demonstrable endocrine disorder. The results lead to the following conclusions.

The posterior lobe of the pituitary exercises the most profound influence on the tolerance; the thyroid a slight influence only but in the same sense as the pituitary, *i.e.* lowered function determines increased tolerance and *vice versa*; the testicle is without influence, while ovarian failure may cause a fall of 50 per cent in the tolerance level. Pluriglandular cases as observed exist only as the result of surgery and show the algebraic sum of the two influences involved. Adrenal failures apparently show a downward tendency. In the group of non-endocrine cases, lesions of the central nervous system, syphilis, malignancy, and anemia seemingly operate to lower the tolerance.

**FURTHER OBSERVATIONS ON THE RELATION OF VITAMIN E TO REPRODUCTION IN RATS ON SYNTHETIC AND MILK DIETS.**

BY H. A. MATTILL AND M. M. CLAYTON.

*(From the Department of Vital Economics, University of Rochester, Rochester, New York.)*

Sterility produced in rats by synthetic rations devoid of vitamin E is identical with that which we observed in animals on lard-containing milk rations. On various modifications of a basal ration containing 18 per cent of casein with starch, lard, salt, yeast, and cod liver oil, females showed resorptions. These ceased when the males were 4 to 5 months of age due to the degeneration of the testes. Externally the animals appeared normal. The inclusion of wheat germ oil in the basal ration temporarily accelerated growth in both sexes but restored fertility only to the females. This restoration was more rapid and the number of young born larger with increasing quantities of the oil (1 to 4 per cent), but lactation was very inadequate even in animals receiving a 5 per cent addition from the age of weaning. The addition of 7.5 per cent of wheat germ (less than 1 per cent of oil) not only restored fertility but secured fair lactation. None of the male animals, with a single exception, was restored by wheat germ or the oil unless these were supplied before the age of 4 to 5 months. Functional tests only can differentiate between prophylaxis and cure. Lard rendered at low temperature was not superior to commercial lard in the basal ration, nor did radiation of the animals or of the basal ration prevent sterility.

**THE RÔLE OF VITAMIN E IN LACTATION.**

BY BARNETT SURE.

*(From the Department of Agricultural Chemistry, University of Arkansas, Fayetteville.)*

Vitamin E regulates two physiological processes. It prevents resorption of the fetus during gestation, and influences lactation after the young are born. As little as 1 mg. per animal per day of a concentrate of unsaponifiable matter from wheat oil was found sufficient to prevent sterility, but none of the normal young born were weaned. About 76 out of 100 young given to mothers to rear have been weaned on 4 mg. per day of a concentrate from

wheat oil (practically free from sitosterol). Reducing the same concentrate to 2 mg. per animal per day, only 9 young out of 60 were weaned, although healthy, normal litters were born. Oxidation by aeration of acetone and ethereal extracts of wheat oil for 24 hours at 90 and 110°C. showed no deleterious effect on fertility but produced considerable mortality of the young during lactation. It is perfectly evident from work so far completed that the reproductive dietary complex, termed vitamin E, contains a hitherto unrecognized lactation factor. Whether or not it is necessary to postulate the existence of a specific vitamin for lactation will become more apparent later.

#### THE CHEMISTRY OF VITAMIN A.

By J. C. DRUMMOND, H. J. CHANNON, AND K. H. COWARD.  
(*From the Biochemical Department, University College, London, England.*)

The unsaponifiable matter of cod liver oil was extracted, dissolved in methyl alcohol, and the greater part of the cholesterol removed by crystallisation at  $-10^{\circ}\text{C}$ . The rest was precipitated out with digitonin. Apparently none of the vitamin was removed by this process. From material obtained in this way it was determined that the vitamin is volatile in steam and that it distils chiefly at a temperature of  $180-220^{\circ}\text{C}$ . under 2 to 3 mm. pressure. It contains only carbon, hydrogen, and oxygen; no nitrogen or iodine. Spinacene, which is not the vitamin, may be identified in this fraction and estimated roughly by precipitating as the hydrochloride or duodecabromide. Allowing for this, the spinacene-free residue containing the vitamin is found to have an iodine value of about 103, an acetyl value of about 215, and a molecular weight of about 300. These figures would suggest that the other main constituent or constituents of the active fractions might be one or more unsaturated alcohols containing one hydroxyl group and one ethylene linkage. Such of the higher alcohols as batyl alcohol, oleyl alcohol, and selachyl alcohol occurring in certain liver oils are without physiological activity. Phytol is also inactive. It is wholly uncertain whether the higher alcohol indicated in the active fraction is the vitamin or whether the vitamin is some other substance occurring in still smaller quantity. It has no aldehydic properties and is not destroyed by acetylation or benzylation.

**A STUDY OF THE VITAMIN A AND B CONTENT OF MIXED HUMAN MILK.**

By ICIE G. MACY, JULIA OUTHOUSE, M. LOUISA LONG, AND B. RAYMOND HOOBLER.

(From the Nutrition Research Laboratories of the Merrill-Palmer School, the Children's Hospital of Michigan, and the Woman's Hospital and Infant's Home, Detroit.)

From biological experiments it has been shown that 10 cc. of human milk daily supplied sufficient vitamin A,<sup>12</sup> whereas a similar quantity may produce fair growth<sup>13</sup> or it may be entirely inadequate for the vitamin B<sup>12</sup> requirement of the rat. Similarly, 20 cc. failed to protect fowls against polyneuritis.<sup>14</sup> Since few data on the vitamin content of breast milk are available, the experimental results obtained in a preliminary investigation are presented.

A daily composite sample of milk from ten to sixteen wet nurses was fed to growing rats receiving a purified basal ration made adequate in respect to all the known dietary essentials with the exception of the vitamin under investigation; this was furnished by the milk. The vitamins and milk were fed fresh daily in separate dishes apart from the dried food mixture. In the vitamin B studies all animals were kept on raised screens. Individual food records were ascertained; daily vaginal smear examinations made; whenever possible, the ability of the female to rear normal young was tested.

In no instance was it possible to secure growth with quantities less than 12 cc. of milk daily as the only source of vitamin B, and it was not until 20 cc. were given that growth approximated the normal; levels of 25 and 30 cc. daily stimulated simultaneously growth and consumption of the basal ration. There was a tendency towards delayed sexual maturity in the females on milk levels below 18 cc.; in some animals normal ovulation took place when a minimum of 20 cc. of breast milk were consumed, whereas others have required 25 cc. and 30 cc.

<sup>12</sup> Kennedy, C., Palmer, L. S., and Schlutz, F. W., *Tr. Am. Pediat. Soc.*, 1923, xxxv, 26.

<sup>13</sup> Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Year Bk. No. 19*, 1920, 389; *Biochem. J.*, 1922, xvii, 363.

<sup>14</sup> Gibson, R. B., *Philippine J. Sc., Sect. B*, 1913, viii, 475.

Experiments indicate that 1 and 1½ cc. of human milk as the only source of vitamin A are inadequate over a long period of time. If the animal body is permitted to retain its vitamin A store, the rat has the ability to attain normal growth on 2 cc., but when the store is first exhausted this quantity is not always sufficient for normal bodily functions. There has been no apparent superiority of 15 cc. over 2 cc., females on a 5 cc. level as well as those on 10 and 15 cc. levels have produced and reared normal young. Reproduction on lower levels of milk for vitamin A as well as for vitamin B is being studied. This preliminary report deals with but one phase of a series of studies on the chemical and biological aspects of human milk production which are now in progress in our laboratories.

#### THE ANTIRACHITIC PROPERTIES OF CERTAIN LIPOIDS.

By ELIZABETH M. KOCH, MEYER H. CAHAN, AND REUBEN G. GUSTAVSON.

*(From the Department of Pharmacology, University of Illinois, Chicago, and the Department of Physiological Chemistry, University of Chicago, Chicago.)*

In October, 1924, an investigation of the antirachitic properties of certain lipoids was begun. In confirmation of the work reported by Hess and Steenbock, cholesterol, radiated dry, was found to be active in preventing rickets in rats fed the McCollum diet, No. 3143. Purified oleic and palmitic acids radiated and added to the food caused a remarkable acceleration in growth followed by severe rickets. Radiated lecithin from brain was inactive except in large amounts. This protective action was probably due to the traces of cholesterol present. Radiated tyrosine also proved of no value.

In the attempt to learn something further of the changes occurring on radiation, a dry sample of cholesterol purified by repeated crystallization from acetone was radiated 1½ hours and extracted with liquid ammonia by the method of Gustavson. A small amount of brown resinous material was separated. Non-radiated cholesterol extracted in a similar manner was completely insoluble.

The brown, liquid ammonia-soluble residue was incorporated

with a weighed portion of food and fed in amounts to give daily doses of 0.25 mg. of residue to each of two rats. A remarkable improvement in the growth and general condition of these rats followed the administration of this material. A second preparation which stood a month after extraction before incorporating with food was entirely inactive in 0.3 mg. doses.

The non-saponifiable fraction of cod liver oil was extracted with liquid ammonia and again a brown, gummy residue was obtained. This fed in daily doses of 2 mg. prevented rickets entirely.

#### A RELATION BETWEEN APPETITE AND THE ENERGY FACTOR IN NUTRITION: A PRACTICAL CONSIDERATION.

By GEORGE R. COWGILL.

(From the Department of Physiological Chemistry, Yale University, New Haven.)

Our feeding experiments on dogs have shown that when a "complete" diet is fed, the animal eats all food offered and gains in weight until a certain maximum is reached; thereafter the dog eats such amounts of food daily as are necessary to maintain the body weight between rather narrow limits. When various diets differing markedly in energy content are fed, it is found that while the animal varies its daily food intake considerably, it maintains over any period of a week or more a caloric intake that is practically constant. In other words the animal *eats calories*. This idea in itself is not new, Rubner<sup>15</sup> having voiced his belief in it; many other students of nutrition also have assumed it to be true.

In metabolism experiments it is frequently desired to maintain a constant daily food intake. This involves among other things the administration of amounts of vitamin B sufficient to maintain the appetite.<sup>16</sup> As the writer has previously pointed out, metabolism animals may fail to eat all of the food offered, not because of any vitamin or other deficiency but because too many calories are being fed. Such an animal owes its *apparent*

<sup>15</sup> Rubner, M., *Die Gesetze des Energieverbrauches bei Ernährung*, Leipzig, 1902, p. lxxxiii; quoted by Lusk, G., *The elements of the science of nutrition*, Philadelphia and London, 2nd edition, 1909, 218.

<sup>16</sup> Cowgill, G. R., *J. Biol. Chem.*, 1923, lvi, 725.



loss of appetite to an adjustment of its energy intake to its energy needs. A control of the energy factor in relation to appetite is obviously desirable.

On the basis of numerous data at hand from feeding experiments carried out during the past 3 years and from the feeding of laboratory animals by students in this laboratory, it has become possible to construct tables of energy intake suitable for dogs fed according to our method described elsewhere<sup>16</sup> and compatible with the maintenance of appetite and excellent physiological condition while living in metabolism cages. In other words a control of the energy factor in relation to appetite for these experimental conditions has been worked out.

#### STUDIES IN METHYLATION.\*

By N JEAN NOVELLO, BENJAMIN HARROW, AND C P SHERWIN.

(From the Research Laboratory of Physiological Chemistry, Fordham University, New York )

In an attempt to study detoxication by methylation *in vivo*, the following substances were fed to dogs and rabbits: imidazole, pyridine, quinoline, piperidine, and quinaldine. Imidazole was largely destroyed in the body, but was not detoxicated by methylation. It yielded ammonia, some urea, but considerable allantoin and uric acid. Pyridine was qualitatively but not quantitatively methylated in the organism of the dog. Piperidine proved extremely toxic, but was to some degree excreted as an ethereal sulfate. The interesting reactions were encountered with quinoline, which was oxidized through the benzene ring yielding a pyridine derivative. Quinaldine on the contrary was oxidized through the pyridine ring, yielding some acetylaminobenzoic acid. Imidazole lactic acid, imidazole pyruvic acid, and imidazole acrylic acid were fed to rats on a histidine-free diet. As reported by W. C. Rose, we also found histidine to be an essential amino acid. Imidazole pyruvic acid is able to some extent to replace histidine in the diet; but imidazole lactic acid is much more efficient in the capacity, while imidazole acrylic acid is less so, but like the others seems to be catabolized in the body much the same as histidine. Imidazole alone has no value when used with a histidine-free diet.

\* Presented before the Society of Pharmacology and Experimental Therapeutics.

**DESTRUCTION OF AN HYDANTOIN NUCLEUS (BETA-METHYL HYDANTOIN) IN THE ANIMAL BODY.\***

By O. H. GAEBLER.

*(From the Biochemical Laboratory, State University of Iowa, Iowa City.)*

In several experiments in which  $1\frac{1}{2}$  to 4 gm. of beta-methyl hydantoin were administered to a dog subcutaneously, it was found that only 15 per cent of the compound was excreted unchanged, and that an equal amount appeared as methyl hydantoic acid. The increase in total nitrogen accounted for all of the hydantoin derivative administered. Creatinine and creatine values show no change when corrected for the amount of beta-methyl hydantoin and methyl hydantoic acid excreted. Allantoin is unaffected. Urea excretion, determined by urease, rises sufficiently to account for 20 per cent of the compound given. Oxalic acid output is increased 50 times. This, with results for amine nitrogen in Kjeldahl distillates, suggests that beta-methyl hydantoin is converted to methyl hydantoic acid, which is then hydrolyzed to glycollic acid and methyl urea, the former being oxidized to oxalic acid, and the latter in part converted to urea.

In the course of preparation of beta-methyl hydantoin from creatinine, experiments on the course of this reaction were carried out. These indicate that the methyl hydantoic acid formed by action of baryta on creatinine originates largely from condensation of sarcosine and urea with elimination of ammonia, rather than directly from creatine, or from creatinine by way of beta-methyl hydantoin.

Beta-methyl hydantoin gives a strong positive reaction when Rothera's nitroprusside test for acetone bodies is applied. Methyl hydantoic acid gives a negative test.

**INTESTINAL ABSORPTION OF CALCIUM AND PHOSPHORUS.\***

By OLAF BERGEIM.

*(From the Department of Physiology and Physiological Chemistry, University of Illinois, College of Medicine, Chicago.)*

A new and simple method is described for the study of absorption from the different parts of the gastrointestinal tract and for the determination of the ultimate utilization or digestibility of

\* Presented before the Society of Pharmacology and Experimental Therapeutics.

foods. The application of the method to the study of the absorption of calcium and phosphorus is discussed and data presented showing the influence of various factors on the absorption of these elements, particularly in rickets.

#### THE EFFECT OF ANESTHESIA ON PHOSPHATE METABOLISM.\*

By ADOLPH BOLLIGER.

*(From the Henry Ford Hospital, Detroit.)*

Phosphate metabolism in dogs, as indicated by studies on blood and urine, has been followed during and after anesthesia produced by ether, chloroform, ethylene, chloretone, urethane, and amytal. In contrast to the work of Potter, Martland, and Robison and Stehle and Bourne it is found that in most instances the anesthetics mentioned depress blood phosphates. This depression starts simultaneously with administration.

Consistent elevation of blood phosphates is coincident with asphyxia, excessive struggling during induction, forced rapid induction, and unusually long deep anesthesia.

The recovery period following anesthesia in these experiments is marked by a depression in blood phosphates to approximately the same low level regardless of the rise or fall during the anesthesia itself. During this recovery period the phosphate content of the blood drops to a level below the kidney threshold so that none is excreted in the urine for a period of an hour or more.

The two common adjuncts of general anesthesia, morphine and atropine, affect the phosphate metabolism, morphine lowering the phosphate level for a period of 1 or 2 hours and atropine producing a slight elevation. After the initial depression from morphine the phosphates rise above the normal level. When used with general anesthesia this period of elevation from the morphine neutralizes to some extent the phosphate depression of the anesthetic.

#### THE PROMOTER ACTION OF ADRENALIN ON PTYALIN.\*

By E. W. ROCKWOOD AND ANNA K. KELTCH.

*(From the Department of Toxicology, State University of Iowa, Iowa City.)*

Digestion was carried on at 38° with varying concentrations of starch and adrenalin, its rate being based upon the reducing sugar

\* Presented before the Society of Pharmacology and Experimental Therapeutics.

produced. The pH was kept constant. When the amount of starch was relatively large there was a marked promoter effect from the adrenalin, in general proportional to the amount of adrenalin present. This was shown by the natural adrenalin, adrenalin hydrochloride, and synthetic adrenalin. While the limits of its activity were not determined its effects were strongly marked at a dilution of over one per million.

With a substrate of liver glycogen adrenalin has a similar effect upon ptyalin. This was not observed, when adrenalin was added to pulped liver tissue, nor with a solution of liver glycogen with pulped liver in the presence of adrenalin.

#### DIHYDROXYACETONE METABOLISM.\*

By W. R. CAMPBELL, A. A. FLETCHER, J. HEPBURN, AND  
J. MARKOWITZ.

*(From the Departments of Medicine and Physiology, University of Toronto,  
Toronto, Canada.)*

The triose dihydroxyacetone has been credited with being metabolized without the use of insulin and, under such circumstances, with possessing antiketogenic powers. It has been assigned a rôle as an intermediary metabolite of glucose. It has not previously been detected in the blood stream even after administration of considerable amounts.

A method of estimation of dihydroxyacetone in solution in the presence of glucose has been devised. It consists in reducing molybdenum in phosphoric acid solution and then reoxidizing with permanganate.

By this method, which detects one part in half a million, no dihydroxyacetone could be found in normal blood. In normals given 100 gm. of dihydroxyacetone by mouth the substance is absorbed and can be detected in the blood stream in considerable amounts.

A number of substances have been shown to cure insulin hypoglycemia. All, however, must be converted into glucose or mannose before they are effective. Insulin hypoglycemia in patients has been cured many times by administration of dihydroxyace-

\* Presented before the Society of Pharmacology and Experimental Therapeutics.

tone by mouth. Improvement is somewhat slower than with glucose.

To avoid the subjective side of the reaction and to study the intravenous administration of dihydroxyacetone in hypoglycemia a liberal overdose of insulin was given intravenously to rabbits. When convulsions appeared 1 gm. of dihydroxyacetone was injected intravenously. There was relief from the symptoms of convulsions and coma but not quite so rapidly as with glucose. The dihydroxyacetone in the blood falls; the total and true blood sugar rises; and the animal remains cured when the triose is given. The additional glucose can come from nowhere but the dihydroxyacetone administered.

In the fasted depancreatized dog dihydroxyacetone given by mouth is recovered quantitatively in the urine as glucose in the same time as if glucose itself were fed. There is no reduction in the total acetone body excretion during the day. The total blood sugar rises, the dihydroxyacetone in the blood increases, then disappears, and the true blood sugar rises parallel to the disappearance of the dihydroxyacetone.

When the glucose in a constant diet and insulin régime is replaced by an equal weight of dihydroxyacetone the glucose output of a depancreatized dog remains the same, thus showing no preferential utilization of dihydroxyacetone over glucose with the same amount of insulin. In the fasting depancreatized dog the respiratory quotient falls after administration of dihydroxyacetone the same as after the administration of glucose.

Some importance has been assigned the phosphates in carbohydrate metabolism. In normal human subjects the administration of equal quantities of dihydroxyacetone and glucose produces identical results on the phosphate curve.

The human diabetic patient behaves similarly to a partially depancreatized animal, utilizing, or not utilizing, the dihydroxyacetone according to his remaining capacity to produce insulin. Where the capacity for stimulation remains the blood sugar may fall; with severe cases, however, addition or substitution of dihydroxyacetone in the diet causes hyperglycemia and glycosuria accompanied by a respiratory quotient at the fat level, as in the fully depancreatized animal, and no improvement in the ketosis occurs. The evidence available, in our opinion, precludes any

possibility of dihydroxyacetone possessing a greater clinical value than other substances, such as glycerol, which are more slowly converted into glucose than the starches. It also appears doubtful that dihydroxyacetone is in the main path of intermediary metabolism of carbohydrate in the body.

#### THE OPTICAL ACTIVITY OF CYSTEINE.

By JAMES C. ANDREWS.

*(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)*

Contrary to statements usually found in the literature, the specific rotation of cysteine prepared from *l*-cystine equals about +9.5. The literature values range from about -12 to -16. These negative values undoubtedly resulted from incomplete reduction. Complete reduction is effected either with metals (Zn, Sn, etc.) in acid solution or by cathodic electrolysis, the latter method being by far the more satisfactory. As the rotations of various samples of cystine change from -220 to 0, the rotations of the corresponding samples of cysteine change from +9.5 to 0.

#### THE CHOLESTEROL CONTENT OF THE CUTANEOUS EPITHELIUM OF MAN.

By H. C. ECKSTEIN AND UDO J. WILE.

*(From the Departments of Physiological Chemistry and of Dermatology and Syphilology, School of Medicine, University of Michigan, Ann Arbor.)*

The cholesterol content of the outer layers of human skin was determined by the digitonin method and found to be very high. The percentage of cholesterol in four normal and six pathological specimens varied from 13.5 to 24 per cent of the total lipoids, but of the samples analyzed eight gave results ranging from 18.6 to 19.5 per cent. Contrasted with this, the cholesterol content in the subcutaneous fat of man was recently reported by one of us<sup>17</sup> to be as low as 0.24 per cent of the total lipoids.

<sup>17</sup> Eckstein, H. C., *J. Biol. Chem.*, 1925, lxiv, 797.

**LACTOSE ESTIMATION UNIFORM IN TECHNIQUE WITH ESTIMATION OF GLUCOSE IN BLOOD AND URINE.**

BY HOWARD D. HASKINS.

*(From the Department of Biochemistry, Medical School, University of Oregon, Portland.)*

Haskins and Holbrook have recently modified the Shaffer-Hartmann method, and have suggested a uniform technique for estimating glucose in both blood and urine, using the micro copper reagent. The present author has extended the method to include the estimation of lactose in urine and milk, using the same reagents and technique. The modification referred to has enabled Haskins to construct a table from which the percentage of sugar (glucose or lactose) in the original blood, urine, or milk is read off opposite the cc. of thiosulfate used for titration. The results by this technique have been proved to be accurate.

**NEW HEMOGLOBINOMETERS.**

BY HOWARD D. HASKINS AND E. E. OSGOOD.

*(From the Department of Biochemistry, Medical School, University of Oregon, Portland.)*

The new permanent standard prepared by Haskins for use with the Sahli apparatus has proved satisfactory and gives accurate estimations. The Osgood-Haskins standard solution for use with the Kober or Duboscq colorimeter has been shown to be absolutely permanent and to give estimations of hemoglobin varying from absolute accuracy by less than 2 per cent.

**THE RATE OF FILTRATION OF SOME AQUEOUS PROTEIN SOLUTIONS.**

BY SAMUEL AMBERG.

WITH THE TECHNICAL ASSISTANCE OF FRANCES SAWYER.

*(From the Department of Pediatrics, Mayo Foundation, Rochester, Minnesota.)*

In order to obtain a fairly accurate method, alundum crucibles were used with a pressure of 30 mm. of mercury. The solutions of proteins tested were gelatin, caseinogen, and mucin prepared according to the Hammarsten method from submaxillary gland of cattle.

It was found that the mucin solution filtered much slower than the gelatin or casein solutions. The rate of filtration is not dependent on the viscosity for the viscosity of blood serum is much greater than that of mucin solutions which filtered slower than blood serum. The rate of filtration does not seem to have any definite relation to the surface tension of the solution as measured with the new Du Noüy apparatus. Comparisons are made with other glucoproteins.

**CURVES OF THE BLOOD AND CEREBROSPINAL FLUID SUGAR  
FOLLOWING GLUCOSE INGESTION IN A CASE OF  
CEREBROSPINAL RHINORRHEA.**

By R. B. GIBSON AND HERMAN DULANEY.

*(From the Clinical Chemistry Laboratory of the Department of Internal  
Medicine, State University of Iowa, Iowa City.)*

Blood and cerebrospinal fluid sugar determinations were made by the Folin-Wu and Benedict methods at half hour periods over periods of 3 hours after the ingestion of 50 gm. of glucose. The cerebrospinal fluid sugar showed similar but less pronounced variations as compared with the blood sugar with a lag of 1 hour when the maximum content was obtained. Micro adaptation of the analytical procedures permitted exact timing of the collection of the fluid.

**THE EFFECT OF PROTEOLYTIC ENZYMES ON THE ACTIVE PRIN-  
CIPLE OF TUBERCULIN.**

By FLORENCE B. SEIBERT AND ESMOND R. LONG.

**AN ACCURATE METHOD OF ESTIMATING UREA IN FOLIN-WU  
BLOOD FILTRATES.**

By L. P. CLARK AND J. B. COLLIP.

**A NEW METHOD FOR THE DETERMINATION OF ALLANTOIN IN  
URINE.**

By A. A. CHRISTMAN.

**SPECIFIC ABSORPTION STUDIES UPON RENNIN.**

By B. E. L. FRENCH.

**IN VITRO STUDIES ON AMMONIA AND UREA FORMATION BY  
TISSUES.**

By H. C. GOLDTHORPE.



**MODIFICATION OF THE TISDALL METHOD FOR BLOOD PLASMA CALCIUM.**

By W. R. TWEEDY.

**THE INFLUENCE OF AN ATHLETIC TYPE OF EXERCISE UPON THE QUANTITY OF UREA ACID, CREATINE, AND CREATININE IN BLOOD AND URINE.**

By WARREN C. WADE.

**THE NUTRITIONAL VALUE OF CHLOROPHYLL AS RELATED TO HEMOGLOBIN FORMATION.**

By CHARLES W. SAUNDERS.

**THE APPLICATION OF OXIDATION-REDUCTION POTENTIALS TO THE MEASUREMENT OF OXIDASE ACTIVITY.**

By MARTIN E. HANKE AND J. A. TUTA.

**A STUDY OF THE BLOOD INORGANIC PHOSPHATE AS VARIED BY RACHITIC AND NON-RACHITIC DIETS.**

By ELIZABETH M. KOCH AND MEYER H. CAHAN.

**STUDIES OF THE EFFECT OF TEMPERATURE ON THE CATALASE REACTION.**

By SERGIUS MORGULIS, M. BEBER, AND I. RABKIN.

**CHEMICAL AND PHYSIOLOGICAL EFFECTS OF IRRADIATION WITH ULTRA-VIOLET LIGHT.**

By H. STEENBOCK, C. A. HOPPERT, AND B. M. RIISING.

**THE SPECIFIC DYNAMIC ACTION OF VARIOUS CARBOHYDRATES IN DOGS.**

By IRENE SANDIFORD AND HARRY J. DEUEL, Jr. (45-)

**THE METABOLISM OF GALACTOSE.**

**IV. THE INFLUENCE OF OVARIAN ACTIVITY ON THE GALACTOSE TOLERANCE.**

By A. W. ROWE.





